# Wnt5a is essential for dendrite maintenance and cognitive functions in the adult brain

By

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## Abstract

Long-term structural maintenance of neuronal networks is essential for sustaining brain functions. The size and pattern of dendrite arbors dictate the ability of neurons to receive and integrate synaptic inputs and are thus critical determinants of information processing in the brain. Established during embryonic and postnatal development, dendrite arbors and many dedritic spines are largely stable in adulthood and are maintained for the lifetime of an organism. Late-onset retraction of dendritic arbors and spine loss are the most consistent morphological correlatlations of common neurological disorders including schizophrenia, chronic stress, anxiety and Alzheimer's disease. Molecular signals that sustain neuronal morphology and synaptic connectivity in the adult nervous system, however, remain largely unknown.

Wnts are secreted glycoproteins that have critical and evolutionarily conserved functions during embryonic development. In the nervous system, Wnt5a has emerged, among the 19 vertebrate Wnts, as a critical regulator of neuronal morphogenesis and synapse formation during development. Interestingly, I found that in the mouse hippocampus, a brain structure critical for the learning and memory, the onset of Wnt5a expression occurs only postnatally. Owing to the early neonatal lethality and global developmental defects in conventional Wnt5a mutant mice, the functions of Wnt5a in the postnatal brain are poorly understood.

Using *Wnt5a* conditioanl knockout mouse model, I investigated the functions of Wnt5a in the adult hippocampus. I reveal an essential role for Wnt5a in maintaining dendrite morphology in the adult hippocampus. Neuron-specific deletion of *Wnt5a* in mice does not compromise dendrite development in CA1 hippocampal pyramidal

neurons, but results in striking adult-onset defects in dendrite arborization, total dendritic lengths and spine densities that manifest after 3 months of age in mice. Electrophysiological analyses revealed impaired synaptic plasticity that preceded the onset of structural alterations in dendrites. Notably, adult Wnt5a mutant animals showed defects in cognitive functions that progressively declined with the manifestation of structural abnormalities. Furthermore, biochemical analyses showed attenuated signaling of key components of calcium and planar cell polarity pathways, and a decrease in CREB-mediated transcription of the obligatory NMDA receptor subunit, GluN1, in the mature Wnt5a mutant hippocampus. These molecular changes could underlie the cognitive and structral abnormalities due to the loss of Wnt5a. Together, this study provide the first direct *in vivo* evidence that Wnt5a is essential for maintenance of neuronal connectivity and for cognitive functions and provide insight into neuroprotective mechanisms in the adult brain.

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# Preface

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## Table of Content

# List of figures

# List of Abbreviations

| | |
|---|---|
| AMPA | α-amino-3-hydroxy-5-metnyl-4-isoazolepropionic acid |
| APC | adenomatous polyposis coli |
| BDNF | brain-derived neurotrophic factor |
| BSA | bovine serum albumin |
| CA1 | cornus ammonis 1 |
| CaMKII | $Ca^{2+}$/Calmodulin-dependent protein kinase II |
| CREB | cAMP response elements binding protein |
| DAG | diacyl glycerol |
| DG | dentate gyrus |
| DKK-1 | dickkopf-related protein 1 |
| DNA | deoxyribonucleic acid |
| Dvl | dishevelled |
| ECS | electro-convulsive shock |
| EE | enriched environment |
| ER | endoplasmic reticulum |
| EPSC | excitatory postsynaptic current |
| ERK | extracellular signal-related kinase |
| FOXY-5 | formylated Wnt-5a-derived hexapeptide |
| Fz | frizzled |
| GABA | γ-aminobutyric acid |
| GFP | green fluorescent protein |
| GluA1 | AMPA receptor subunit 1 |

| | |
|---|---|
| GluN1 | NMDA receptor subunit 1 |
| GluN2A | NMDA receptor subunit 2A |
| GluN2B | NMDA receptor subunit 2B |
| GSK-3β | glycogen synthase kinase 3β |
| GTPase | guanosine triphosphatase |
| HEK293 | human embryonic kidney 293 |
| HEPES | N-(2-hydroxyethyl)piperazine-N-(2-ethanesulfonic acid) |
| Hz | hertz |
| IP3 | inositol 1,4,5-trisphosphate |
| JNK | c-JunN-terminal kinase |
| KCl | potassium chloride |
| LRP5 | the low-density lipoprotein receptor related protein-5 |
| LRP6 | the low-density lipoprotein receptor related protein-5 |
| LTD | long-term depression |
| LTP | long-term potentiation |
| mEPSC | miniature excitatory postsynaptic current |
| MF | mossy fiber |
| ml | milliliter |
| mM | millimolar |
| mRNA | messenger RNA |
| ms | millisecond |
| mV | millivolt |
| NFAT | nuclear factor of acativated T cells |

| | |
|---|---|
| NF-κB | nuclear factor kappa-light-chain-enhancer of activated B cells |
| NMDA | N-methyl-D-aspartate |
| OB | olfactory bulb |
| PBS | phosphate-buffered saline |
| PCP | pathway-the planar cell polarity |
| PCR | polymerase chain reaction |
| PDZ | postsynaptic density-95/discs large/zona occudens-1 |
| PFA | paraformaldehyde |
| PKA | protein kinase A |
| PKC | protein kinase C |
| PLC | phospholipase C |
| PSD | postsynaptic density |
| PSD-95 | postsynaptic density protein of 95 kDa |
| PTEN | phosphatase and tensin homolog |
| qPCR | quantitative real-time PCR |
| ROCK | Rho-associated protein kinase |
| SEM | standard error of the mean |
| TBS | Tris-buffered saline |
| TBST | TBS-Tween 20 |
| TDBL | total dendritic branch length |
| TrkB | tropomyosin receptor kinase B |
| TTX | tetrodotoxin |
| WASP-1 | Wnt signaling potentiator-1 |

# Chapter 1: Introduction

Neurons are highly polarized cells that consist of soma, axons and dendrites. These unique structures allow neuronal activity to be transmitted from axons of an upstream neuron to dendrites of downstream neurons via specialized structures called synapses. Neuronal activity is a key player in regulating neuronal development and maintaining brain circuits. It not only locally determines the synaptic strength, regulates stability and plasticity of synapses, dendrites and axons but also controls gene expression that can have a global effect on the entire neuron (Hua and Smith, 2004; McAllister, 2007). The local regulation of synaptic funcion by neuronal activity involves synaptic vesicle release in the pre-synaptic terminal as well as scaffold protein and neurotransmitter receptors trafficking in the post-synapse sites (McAllister, 2007; Newpher and Ehlers, 2008; Saneyoshi et al., 2008). Interestingly, recent studies have shown that neuronal activity regulates the release and expression of Wnts (Ataman et al., 2008; Gogolla et al., 2009; Sahores et al., 2010; Wayman et al., 2006). Moreover, Wnts, Wnt receptors and their downstream effectors are found to modulate various aspects of neuronal funcion including synaptic vesicle release and formation of synapses in several species during animal development (Cerpa et al., 2008; Lucas and Salinas, 1997; Packard et al., 2002).

**Wnts , Wnt receptors and their signaling**

Wnts are first identified as early morphogen that play key roles in various aspects of animal development such as cell fate determination, proliferation, differentiation and embryonic patterning (Angers and Moon, 2009; Davidson and Niehrs, 2010; Petersen and Reddien, 2009). Wnts are secreted proteins belonging to a family with 19 highly

conserved members. Importantly, Wnts have been shown to bind to a variety of receptors and co-receptors in different type of tissues and during different time of development, thus are not surprisingly involved in the initiation of multiple signaling pathways during development (Nusse and Varmus, 2012; van Amerongen and Nusse, 2009). In the pass decade, Wnt signaling cascades were found to be critical for several developmental processes in the nervous system, such as neural tube patterning, neuronal differentiation, axon guidance, dendritic development, and synapse formation (Fradkin et al., 2010; Hur and Zhou, 2010; Korkut and Budnik, 2009; Salinas and Zou, 2008). Genetic mutations in Wnts and Wnt receptors their downstream effectors have been repeatly identified in many pathological diseases such as cancer, late-onset Alzheimer's disease, Familial Exudative Vitreoretinopathy, bone density defects, and Type II diabetes (Liu et al., 2014; Milat and Ng, 2009; Nikopoulos et al., 2010; Welters and Kulkarni, 2008).

Wnt signalings are extremely complex. In addtion to the 19 conserved Wnts, various Wnt receptors and co-receptors also contribute to the diversity of Wnt signalings. Frizzled (Fz), seven-pass transmembrane protein receptors, are the most well known receptors for Wnt ligands and includes a family of 10 frizzled family receptors. Fz receptors can function alone or cooperated with other co-receptors, the low-density lipoprotein receptor related protein-LRP5 or LRP6, to initiate various Wnt signaling pathways (Angers and Moon, 2009; MacDonald et al., 2009; van Amerongen and Nusse, 2009). Besides Fz family receptors, Wnts also interact with tyrosine kinase receptors-Derailed (Drl)/Ryk and Ror (van Amerongen and Nusse, 2009). Through binding with different receptors, Wnts activate distinct signaling cascades. These cascades can be

largely classfied as the canonical (β-catenin) pathway and the non-conical pathway, which is further sub-divided into the planar cell polarity (PCP) and the calcium pathways.

In the canonical signaling pathway, Wnts bind to Fz receptors and their co-receptors (LRP5 or LRP6) to recruit the scaffold protein dishevelled (Dvl) in inhibiting the Glycogen synthase kinase 3β (GSK-3β) activity (Angers and Moon, 2009; Amerongen and Nusse, 2009). This inhibition of GSK-3β subsequently relives β-catenin from protein degradation and allows it to translocate to the nucleus, where it can activate transcritions of many target genes through its interactions with other transcription factor- TCF /LEF.

In the PCP pathway, one of the non-canonical pathway, Wnt signalings are initiated by Fz receptors and their downstream effector- Dvl, which activate the small GTPases, Rac1 and RhoA, and subsequently lead to the activations of c-Jun-N-terminal kinase (JNK) and Rock signaling cascades. The other non-canonical pathway, the calcium pathway, Wnt singlaing is also initiated by Fz and Dvl but activates phospholipase C (PLC), which hydrolyzes phospholipid and converts it to diacyl glycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). The IP3 then activates Inositol trisphosphate receptor, a calcium channel on the ER membrane and induces calcium release from the ER storage. The increase in intracellular calcium concentration then activates various important protein kinases including protein kinase C, calcium–calmodulin kinase II (CaMKII) and calmodulin (Kohn and Moon, 2005). As a result, calcium pathway signaling has been shown to play important roles in the regulation of neuronal plasticity, cytoskeleton organization and activation of gene transcription (Kohn and Moon, 2005).

**Wnt machinery expression in the brain**

The expression of Wnts and other proteins involved in Wnt signaling machinery have been identified in many brain regions including cerebral cortex, olfactory bulb, hippocampus, neocortex and thalamus (Shimogori et al., 2004). Although Wnts are identified as early morphogen that play important roles during animal early development, Wnt1, Wnt5a, and Wnt7a are also expressed in postnatal stage in areas, such as the olfactory bulb, piriform cortex and other olfactory related areas, and are continuously expressed till adulthood (Shimogori et al., 2004). Additionally, several Wnts are detected in young adult rodents, including Wnt1, Wnt2, Wnt4, Wnt5a, Wnt7a, Wnt7b, Wnt8b and Wnt11 (Cerpa et al., 2008; Shimogori et al., 2004; Wayman et al., 2006). Interestingly, the transcript levels of some Wnts are particularly enriched in olfactory bulb and dentate gyrus (DG) in the hippocampus where the neurons have ability to generate from neural stem cell. Expressions of Wnt5a, Wnt7a and Wnt8b, for example, are specifically enriched in the layers of DG, suggsting their function in neurogenesis. In the neocortex, expression patterns of some Wnts aprear to be layer- and region-specific (Shimogori et al., 2004). For example, Wnt2b expression is enriched in layer 4 and 6 of the somatosensory cortex, and in layer 6 of the visual and auditory cortex in young adulthood. Both Wnt5a and Wnt7a transcripts are also detected as layer-specific in the cortices, including prefrontal, parietal and temporal neocortex (Shimogori et al., 2004). Moreover, Wnts and Wnt signaling components are expressed in the thalamus, a region that relays most of the sensory and motor signals to the cortex. Because sensory and motor inputs are constantly changed and need to be modulated, Wnt signaling may have important function in regulating activity and connectivity between thalamus and cortex.

Fz receptors, the major Wnt receptos, are expressed in the brain with interesting spatial and temporal patterns. Fz3 expresses in the olfactory system only from postnatal-day (P) 0 to P7, but its transcript is detected ubiquitously in the hippocampal pyramidal cell layer, DG granular cells and preiform cortex until adulthood (Shimogori et al., 2004). Fz7 expression is detected in the pyramidal cell layer of hippocampus but there is no postnatal expression of Fz7 in the neocortex (Shimogori et al., 2004). Other Fz receptors, Fz1, Fz2, Fz8, Fz9 and Fz10, are ubiquitously expressed in the neocortex and hippocampus until young adulthood. Fz1, Fz2 and Fz9 expression patterns are correlated with Wnt5a expression in OB and DG. Fz4, Fz5 and Fz6 show postnatal expression only in the thalamus but can not be detected in other region postnatally (Shimogori et al., 2004) . In summary, these distinct expression patterns of Wnts and their receptors in the brain suggests that Wnt pathways might be involved in neurogenesis (OB and DG), sensory and motor processing (thalamus) and cognitive functions (cortex and hippocampus) in different brain areas.

**Wnt signaling in the central synapse development**

In the central nervous system (CNS), Wnt and their correlated receptors signaling through pathways that have been suggested to promote synapse formation and synaptic function (Cerpa et al., 2008; Farias et al., 2009; Packard et al., 2002). The first identified Wnt involved in the central synapse development is Wnt7a, which is secreted from cerebellar granule cells to promote synapsin 1, a presynaptic marker, clustering in cultured neurons (Lucas and Salinas, 1997). Further *in vivo* evidence showed that Wnt7a regulates the formation of the mossy fiber and granule cell synapse in the cerebellum

(Hall et al., 2000). Moreover, applying Wnt7a, Wnt3a or Wnt3b, Wnts that are involved in the canonical pathway, independently into cultured hippocampal neurons have been reported to increase synaptogenesis (Cerpa et al., 2008; Sahores et al., 2010; Varela-Nallar et al., 2010b). Furthermore, Wnt5a, via the non-canonical pathway, has been shown to promote PSD-95, major postsynaptic scaffold proteins at excitatory synapses, clustering and stimulate synapse formation (Farias et al., 2009; Varela-Nallar et al., 2010a). On the other hand, Wnt5a also regulates $GABA_A$ receptors recycling (Cuitino et al., 2010). Wnt8a works through canonical pathway to promote synaptogenesis by activacting receptor Fz/LRP6 (Sharma et al., 2013). Together, these studies suggest that both canonical and non-canonical Wnt signalings play critical roles in pre- and post-synaptic structure and function in the brain.

**Role of Neuronal Activity in Wnt-Mediated synaptic and dendrite Morphogenesis**

Neuronal activity is a key regulator of synaptic and dendritic development (Heiman and Shaham, 2010; Hua and Smith, 2004). In the CNS, neuronal activity has been shown to remodel dendritic spines in enlarging the size of the spine head and shortening the length of the spine neck (Bosch and Hayashi, 2012). Elevation of neuronal activity by KCl-mediated neuronal deplorization increases dendritic arborizations of cultured hippocampal neurons through the β-catenin-dependent canonical pathway (Yu and Malenka, 2003). In contrast, applying Dkk-1, a Wnt antagonist, showed decreased dendritic branches after KCl stimulation (Yu and Malenka, 2003). In addition, Wnt7b and Dvl1 have also been shown to promote dendritic branching through the downstream Rac1 and JNK signaling *in vitro* (Rosso et al., 2005). Interestingly, expression or secretion of

Wnts are often associated with neuronal activity. For example, neuronal activity has been shown to increase Wnt2 gene expression, via the CREB-dependent transcription, to drive dendritic outgrowth in culture hippocampal neurons (Yu and Malenka, 2003). Another independent research group found that KCl activated signaling cascades including NMDARs, CaMKK, CaMKI, MEK/ERK and Ras, and in turns enhances Wnt2 transcription via CREB activation (Wayman et al., 2006). Working as feedback loop, many Wnt members' transcripts such as Wnt2, Wnt5a, Wnt7a and Wnt7b are also up-regulated by neuronal activity.

The first *in vivo* evidance to link Wnt signaling and neuronal activity is from a study in the adult hippocampus. Raising adult mice in an enriched environment (EE), a paradigm to naturally enhance neural activity, induces an increase in the remodeling of mossy fiber terminals, spine densities, and synapse number (Gogolla et al., 2009). In the same EE paradigm, Wnt7a/b transcripts were increased in hippcampal CA3 region. In addition, Wnt7a application alone mimics the effects of EE on the spine density and mossy fiber terminal remodeling. Collectively, these results demonstrate that Wnt signaling correlates with neuronal activity to regulate synapse formation in the hippocampus.

**Wnt signaling in neuronal function: From activity to plasticity**

Neuronal activity-dependent secretions of Wnts also regulate synaptic efficacy and plasticity. Patterned activity introduced by electrical stimulations on acute brain slices is known to induces long-lasting increase in synaptic strength (long-term potentiation, LTP) or the opposite (long-term depression, LTD). Interestingly, LTP induction changes

transcripts of Wnt protein and downstream effectors such as Wnt3a, Fz4, β-catenin and Dvl3 (Chen et al., 2006). Moreover, LTP induces the translocation of β−catenin from cytoplasm to the nucleus and leads to the initiation of several Wnt-targetted gene transcriptions (Chen et al., 2006). This evidence shows that increased activity by LTP stimulation can activate Wnt/β−catenin/TCF pathway in hippocampus. On the other hand, activation of Wnt signaling also increases synaptic transmission and facilitates LTP in hippocampal brain slices and in cultured neurons (Budnik and Salinas, 2011; Inestrosa and Arenas, 2010). These studies indicate that Wnt signaling plays important roles in the regulation of spontaneous and activity-dependent neuronal funciton. However, little is know about physiological functions of Wnt signalings in live animals. Recent evidence showed that animals trained in the hidden platform Morris water maze (MWM) showed increased Wnt7a transcript in the hippocampus compared to animals trained in the visible platform. Surprisingly, the expression of Wnt7a transcripts are enriched specifically in the granule cells of DG after training (Tabatadze et al., 2012). Similarly, Wnt5a expression is also increased in the same learning and memory task. Interestingly, mice perfused with either Wnt signaling potentiator-1 (WASP-1), an activator of Wnt/β-catenin pathway (Beaumont et al., 2007), or the formylated Wnt5a-derived hexapeptide (FOXY-5), an activator of both Wnt/JNK and Wnt/$Ca^{2+}$ signaling (Safholm et al., 2008), showed improved performance in the MWM test and in the novel object recognition task (Vargas et al., 2014). Together, these results indicate that both canonical and non-canonical Wnt pathways are important for animal cognitive function.

# Chapter 2: Postnatal expression and activity-dependent regulation of Wnt5a

## Introduction

The breakthroughs in molecular and genetic techniques over the last 30 years have facilitate our understanding on how environment stimulations modulate and shape the brain circuitry. Sensory inputs transformed into neuronal activity which has been shown to play important roles in the formation and refinement of neuronal connectivity by modulating the number, morphology and plasticity of synapses- the junction between pre-synaptic axons and post-synaptic dendrites. Additionally, many activity-dependent genes are identified along with the discovery of their essential roles in regulating not only the neuronal transmission, but also higher brain functions such as learning and memory. Malfunctions of these genes, for example activity-dependent transcription factors, are often found to be associated with cognitive deficits in human and animal models (West and Greenberg, 2011).

Accumulating evidence provides a strong link between neuronal activity and Wnt signaling in many aspects. First, increased neuronal activity, for example with KCl-mediated neuron depolaration, induces the release and/or expression of Wnt proteins (Inestrosa and Arenas, 2010; Yu and Malenka, 2003). Second, activation of NMDA receptors by neuronal activity can trigger CREB-dependent transcription, and in turn up-regulates Wnt expression. Activity-upregulated Wnt2 signaling, for example, is reported to be crucial to promotes activity-dendritic arborization in hippocampal neurons (Yu and Malenka, 2003). Further *in vivo* evidence showed that activity-dependent Wnt signaling regulates synaptic connectivity in the hippocampus of behaving animals. Mice raised in an enriched environment (EE) showed enhanced Wnt7a/b expression in CA3 region which correlates with the increased remodeling of mossy fiber terminals, spine densities,

and synapses number in the same region (Gogolla et al., 2009). Importantly, brain slices treated with Wnt7a showed similar effects in changing the number of synapses and remodeling mossy fiber terminals as EE, providing a strong evidance for the activity-dependent Wnt funcion in synaptic connectivity and neuronal morphology. Together, these finding suggests that neuronal activity regulates neuronal morphology by modulating the expression and/or secretion of Wnts.

## Result

Using *in situ* hybridization and immunoblotting, we analyzed expression of Wnt5a in the mouse hippocampus at time points ranging from embryonic day 18 (E18) to 6 months of age (Figures 2.1 and Figure 2.2). We observed Wnt5a expression starting at 1 week after birth, and the expression level increased prominently by 2 weeks of age and was sustained at adult stages. *Wnt5a* mRNA was localized throughout the hippocampal formation and was enriched in the dentate gyrus (DG) and the Cornu Ammonis (CA)1 region. This expression pattern in the hippocampus is consistent with previous findings (Shimogori et al., 2004), and that in Allen Brain Atlas (http://www.brain-map.org/) where Wnt5a expression has also been noted in the cerebellum and to a lesser degree in the cerebral cortex and olfactory bulb in the adult mouse brain. That prominent Wnt5a expression in the hippocampus is detected only at postnatal stages is intriguing given the reported roles of Wnt5a in embryonic processes in the brain (Blakely et al., 2011; Boitard et al., 2015; Keeble et al., 2006; Li et al., 2009; Zhang et al., 2007). We also found that the onset of Wnt5a expression in hippocampal neurons correlated with the appearance of many pre- and post-synaptic proteins (Figure 2.2).

Our observation that Wnt5a expression is coincident with synaptic maturation and recent studies that expression and/or release of certain Wnt proteins are enhanced by activity-dependent mechanisms in acute adult brain slices or in response to sensory or learning experience *in vivo* (Chen et al., 2006; Gogolla et al., 2009; Wayman et al., 2006) prompted us to take a closer look at the role of neuronal activity in regulating Wnt5a expression. Indeed, we observed that subjecting mice (2 months old) to electro-convulsive shock, a procedure that globally increases neural activity (Cole et al., 1990),

resulted in a 1.65 fold increase in the levels of Wnt5a protein and 2.14 fold increase in transcripts in the hippocampus after 1 hr, and which persisted up to at least 4 hr following electro-convulsive shock treatment (Figures 2.3 A and B; Figures 2.4). Up-regulation of the transcript and protein levels of the immediate early gene *Arc*, known to be regulated by neuronal activity (Lyford et al., 1995), was used to assess the effectiveness of the electro-convulsive paradigm (Figures 2.3 A and B; Figures 2.4). We then check Wnt5a protein level in other brain regions –cortex and cerebellem with the same electro-convulsive shock procotol and find Wnt5a protein levels also up-regulated by activity (Figures 2.5). Furthermore, in cultured rat hippocampal neurons, the activity blocker tetrodotoxin (TTX) significantly reduced Wnt5a protein levels, suggesting that Wnt5a expression is also influenced by spontaneous activity (Figures 2.6 A-D). Conversely, treatment with the $GABA_A$ receptor blocker, bicuculline, or potassium chloride (KCl)-mediated depolarization, that enhance neuronal activity resulted in increased Wnt5a levels (Figures 2.6 A-D). Together, these results suggest that Wnt5a expression is regulated by neuronal activity.


**Figure 2.1: Wnt5a transcript is detected in postnatally, but not prenatally, in the hippocampus.**

*In situ* hybridization shows *Wnt5a* transcript in the mouse hippocampus at postnatal stages. *Wnt5a* is absent at the day of birth (P0) but is detected by 1 week and is abundant in the 3 month old hippocampus. The sense control is shown in the last panel. Scale bar: 300□m.

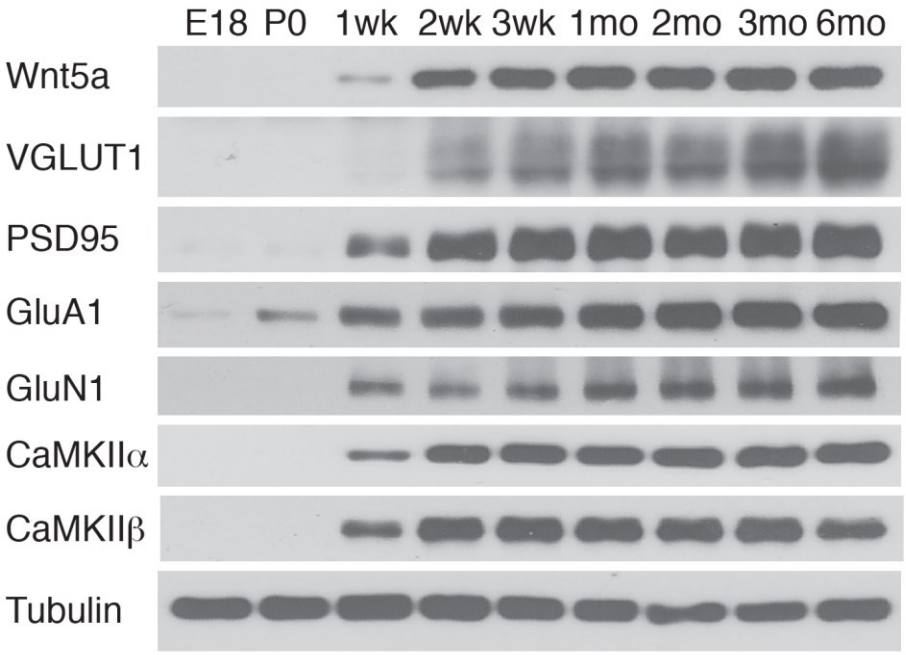



**Figure 2.2: Wnt5a protein expression is detected 1 week after birth, peaks at 4 weeks and persists to adulthood.**

Onset of Wnt5a protein expression coincides with the appearance of pre- and post-synaptic proteins in hippocampal neurons. Hippocampal homogenates from mice of different ages (ranging from E18 to 6 months) were probed using antibodies against Wnt5a, VGLUT1, PSD95, GluA1, GluN1, CaMKIIα, CaMKIIβ and tubulin in immunoblotting analyses.

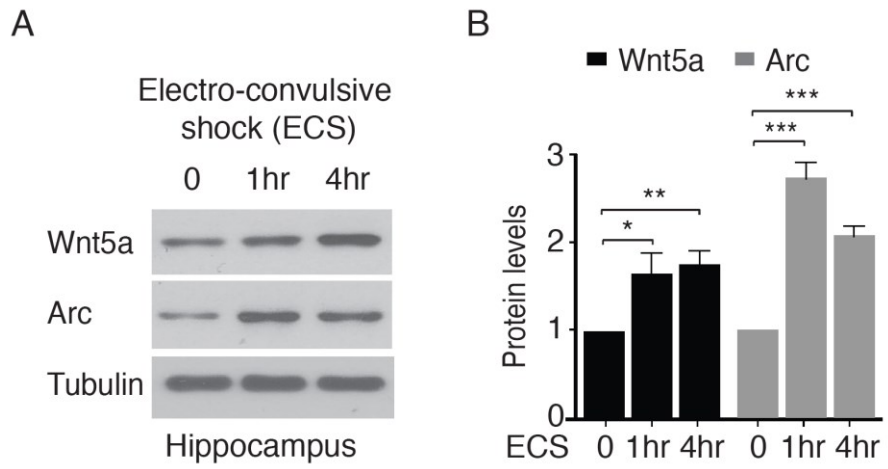



**Figure 2.3: Electro-convulsive shock paradigm up-regulates Wnt5a protein expression in the mice hippocampus.**

**(A)** Electro-convulsive (ECS) shock treatment enhances Wnt5a and Arc protein levels in the hippocampus. Immunoblots were stripped and reprobed for tubulin to account for protein normalization. **(B)** Densitometric quantification of Wnt5a and Arc protein levels. Results are mean ± SEM from 6 independent experiments. Results are mean ± SEM from 6 independent experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$, one-way ANOVA followed by Tukey's post-test.

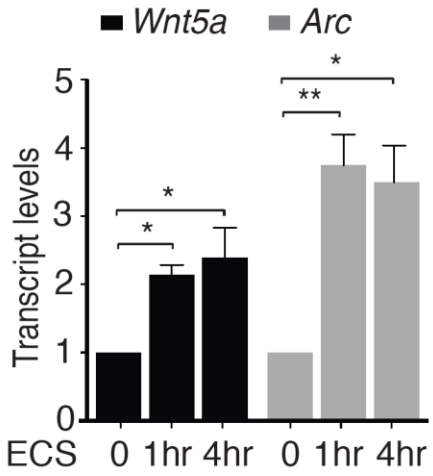



**Figure 2.4: Electro-convulsive shock paradigm up-regulates Wnt5a transcript expression in the hippocampus.**

qPCR analyses show increased *Wnt5a* and *Arc* transcripts in hippocampal lysates with electro-convulsive shock. Results are mean ± SEM from 7 and 4 independent experiments for *Wnt5a* and *Arc*, respectively. *p<0.05, **p<0.01, ***p<0.001, one-way ANOVA followed by post-hoc Tukey's test.

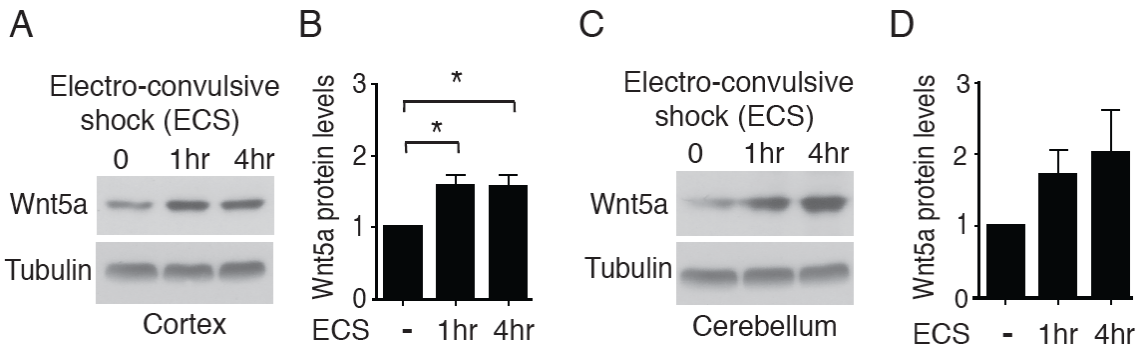



**Figure 2.5: Electro-convulsive shock paradigm up-regulates Wnt5a protein expression in the cortex and cerebellum.**

**(A)** Electro-convulsive (ECS) shock treatment enhances Wnt5a protein levels in the cortex. Immunoblots were stripped and reprobed for tubulin to account for protein normalization. **(B)** Densitometric quantification of Wnt5a protein levels. Results are mean ± SEM from 7 independent experiments. *$p<0.05$, one-way ANOVA followed by Tukey's post-test. **(C)** Electro-convulsive (ECS) shock treatment enhances Wnt5a protein levels in the cerebellum. Immunoblots were stripped and reprobed for tubulin to account for protein normalization. **(D)** Densitometric quantification of Wnt5a protein levels. Results are mean ± SEM from 3 independent experiments.

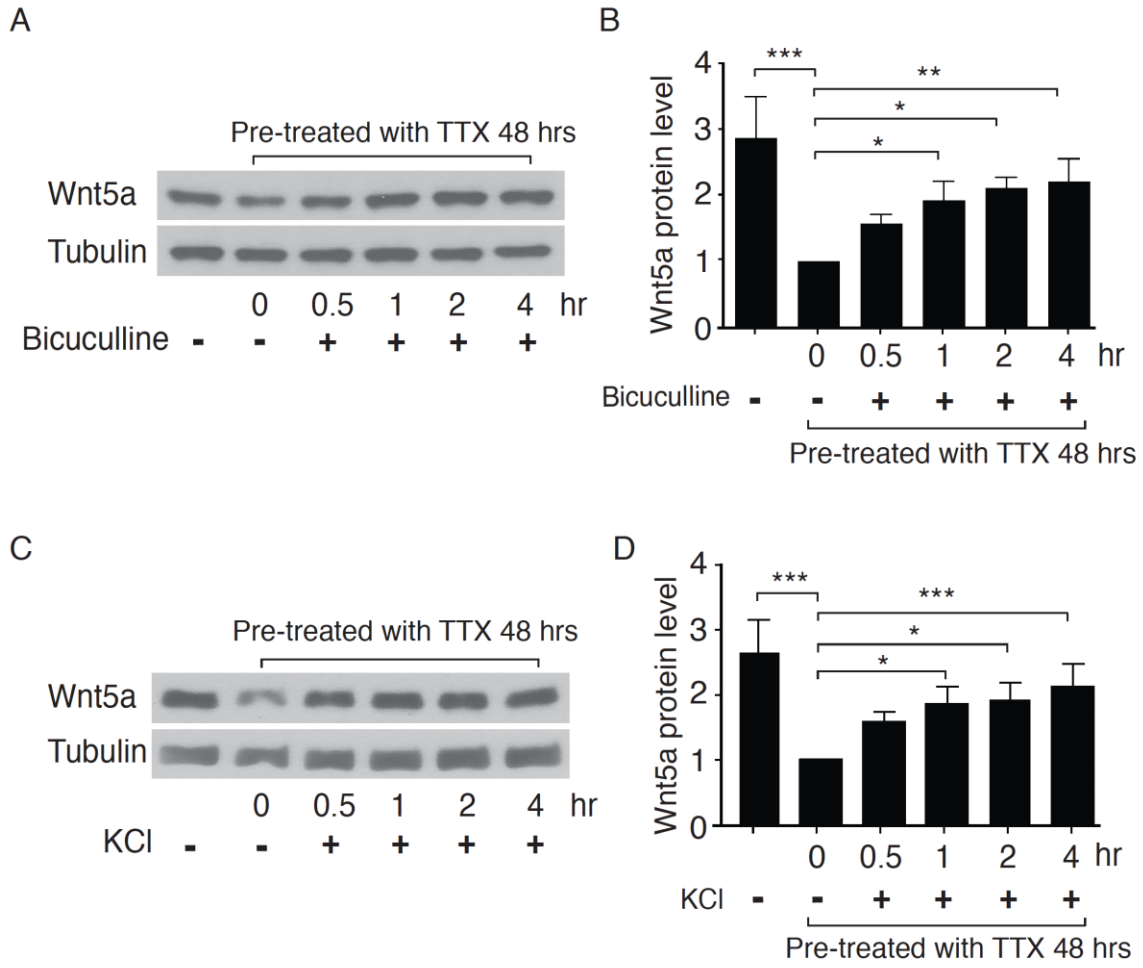



**Figure 2.6: Wnt5a protein expression is regulated by neuronal activity in primary hippocampal neurons.**

**(A-D)** Treatment of rat hippocampal neuron cultures with the activity blocker tetrodotoxin (TTX) significantly reduced Wnt5a protein levels (compare lanes 1 and 2 on Wnt5a immunoblots in A, C, and quantifications in B, D). Wnt5a protein levels subsequently increase after TTX is washed away, and neurons are treated with the $GABA_A$ receptor blocker, bicuculline (40 μM) (A, B), or depolarizing neurons with KCl (20 mM) (C, D) for the indicated time points. Blots were reprobed with tubulin for protein normalization. Densitometric quantification of Wnt5a protein after treatments are

shown in (B, D). Results are mean ± SEM from 5 and 11 independent experiments for (B) and (D), respectively. *$p<0.05$, **$p<0.01$, ***$p<0.001$, one-way ANOVA followed by Tukey's post-test.

## Disscusion

Wnts are identified as morphogens that controlling axis foramation and cell fate determintation during early animal development. Conversely, we found that Wnt5a transcript and protein levels are not detable until the postnatal day 7 in the mouse hippocampus. Wnt5a is profound expressed around 1 month old age and last to the adulthood. In the central nervous system, neuronal activity plays important rores in regulating neuron outgrowth, synaptic function and circurity formation. Here we found that Wnt5a expression level can be up-regulated by neuronal activity in primary hippocampal cultures. Moreover, applying electro-convulsive shock to stimulate brain activity in mice also elevated the Wnt5a transcripts and protein levels in the hippocampus, cortex and cerebellum. These results indicate that *Wnt5a* is an activity-dependent regulated gene at the transcription level.

Calcium signaling is a key mechanism underlying activity-dependent gene regulation in neurons. At excitatory synapses, neuronal activity leads to $Ca^{2+}$ influx largely via N-methyl-D-aspartic acid (NMDA)-type glutamate receptors and L-type voltage-gated $Ca^{2+}$ channels (L-VGCCs) which then trigger a series of intracellular signaling events. Therefore, in the furture we will further investigate the involvement of NMDA receptors and L-VGCCs in regulating Wnt5a expression by using established pharmacological blockers (MK801, APV and nifedipine). Interestingly, in contrast to CREB, the “usual suspect” transcriptional regulator of NMDA-mediated signaling, former graduate student Dr. Daniel Bodmer from our laboratory has found that a dominant negative form of the transcription factor, serum response factor (SRF) selectively attenuated *Wnt5a* levels in sympathetic neurons. Consistently, sequence

analyses of promoter region of *Wnt5a* revealed two consensus SRF binding sites. Furthermore, SRF has been implicated in activity-dependent transcriptional changes in hippocampal neurons. Thus, it would be interesting to employ SRF conditional mutant mice to assess the effect of SRF deletion on Wnt5a expression *in vivo*

## Methods

### Animals

All procedures relating to animal care and treatment conformed to institutional and NIH guidelines. Animals were housed in a standard 12:12 light-dark cycle. Sprague Dawley rats were purchased from Charles River. Hippocampal neuron cultures were established from embryonic day 18 (E18) rat pups as previously described (Araki et al., 2015)

### *In situ* hybridization

*In situ* hybridization was performed using a digoxigenin labeled probe spanning a 572 bp region within *Wnt5a* exon 2. Mouse brains of various ages were fresh frozen in OCT (Tissue-Tek) and serially sectioned (20μm) using a cryostat. Sections were post-fixed in 4% paraformaldehyde (PFA), washed in PBS and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine with 0.9% NaCl. After hybridization with the labeled RNA probe (2 □g/ml) at 68°C overnight, sections were washed with 0.2X SSC buffer at 65°C, blocked with TBS containing 1% normal goat serum and then incubated with alkaline phosphatase-labeled anti-DIG antibody (1:5000; Roche) overnight at 4°C. The alkaline phosphatase reaction was visualized with NBT/BCIP, rinsed in PBS, fixed in 4% PFA and mounted in AquaMount (EMD Chemicals).

### KCl-mediated depolarization or bicuculline treatment of cultured hippocampal neurons

Hippocampal neurons were harvested from E18 Sprague-Dawley rats and grown in poly-L-lysine coated 12 well plates. Cells were maintained in plates with Neurobasal media supplemented with B27, penicillin/streptomycin (1U/ml), and GlutaMax. Cultured

hippocampal neurons were treated with TTX (1 μM/ml) for 48 hr on d.i.v 18 to silence neuronal activity, then TTX was washed away using several washes with Neurobasal culture media, and neurons were then stimulated with either KCl (20mM) or Bicuculline (40 μM/ml) for different times.

**Electro-convulsive shock (ECS)**

Electro-convulsive shock was administered in 8 week-old male *C57BL/6* mice as previously described (Ramanan et al., 2005). Following attachment of saline-soaked ear clips to the mice, 15mA electric pulses (100 Hz, 0.4 ms/ pulse) were delivered through mouse brains for 1 sec using the Ugo Basile ECT unit, Model 57800. Hippocampal tissues were harvested at 1 hr or 4 hr after ECS treatment, and subjected to qPCR or immunoblotting analyses for Wnt5a and Arc. Total protein concentrations were estimated using the BCA assay.

**Statistical analyses**

All Student's *t* tests were performed using two-tailed, unpaired, and a confidence interval of 95%. One-way or two-way ANOVA analyses were performed when more than two groups were compared. Statistical analyses were based on at least 3 independent experiments, and described in the figure legends. All error bars represent the standard error of the mean (s.e.m).

# Chapter 3: The essential roles of Wnt5a for neuronal morphologies in adult hippocampus

## Introduction

In the past decade, studies have revealed that Wnt signaling is critically involved in many key processes in the CNS, including neurogenesis (Lie et al., 2005), synaptic plasticity (Oliva et al., 2013; Tang, 2014), axon guidance (Bodmer et al., 2009; Onishi et al., 2014) and dendritic development (Rosso et al., 2005; Yu and Malenka, 2003). Although most of these processes occur during early neuronal development, the maintenance of neuronal structural is also essential for sustained brain connectivity and brain function in the adult brain. The size and complexity of dendrite structure define the ability of neurons to receive and process synaptic inputs, therefore are imoportant factors for the brain to process information. After an active growing phase during development, dendrite arbors and spines denisties are relative stable and maintained in the adulthood (Koleske, 2013). Many neurological disorders including schizophrenia, chronic stress, anxiety and Alzheimer's diseases have been strongly associated with late-onset decreases in dendritic structure complexity and/or loss of spine number (Anderton et al., 1998; Broadbelt et al., 2002; Kaufmann and Moser, 2000; Kulkarni and Firestein, 2012). Thus, there must be specific molecular signalings to maintain neuronal morphology and synaptic connectivity in the adult nervous system. Currently, little is known about dendrite support mechanisms in adult neurons. Of note, CA1 pyramidal neurons in the hippocampus are particularly vulnerable in Alzheimer's Disease and exhibit extensive dendrite arbor loss that correlates with the degree of cognitive decline (Flood, 1991). Identification of adult maintenance mechanisms would be highly relevant to the understanding of the structural bases of animal behaviors, as well as the etiology of

neurodegenerative diseases where extensive dendritic anomalies are manifested late in life.

Wnt5a is a member of the Wnt protein family and plays important roles in neuronal structure and fucnion, for example axon guidance and branching in both dopaminergic neurons and sympathetic neurons; neurogenesis in dopaminergic neuron; and synapse maturation in primary hippocampal culture neurons (Blakely et al., 2011; Bodmer et al., 2009; Castelo-Branco et al., 2006; Farias et al., 2009). These evidences prompt us to analyze the Wnt5a expression pattern in the CNS specifically in hippocampus. In the present study, we addressed the *in vivo* role of Wnt5a, in the hippocampus, using conditional mutant mice. Contrary to the prevailing view, based largely on *in vitro* studies, we show that Wnt5a is dispensable for the establishment of neuronal connectivity in the postnatal hippocampus *in vivo*. Furthermore, we provide evidence that Wnt5a controls the maintenance of dendrite arborization and spine densities necessary for cognitive functions in adult animals.

## Result

### Wnt5a is necessary for maintenance of dendrite arbors and spine densities in adult neurons

The intriguing spatio-temporal pattern of Wnt5a expression in the postnatal hippocampus, together with recent evidence supporting autocrine actions of Wnts in regulating morphological changes in cultured hippocampal neurons (Rosso et al., 2005; Wayman et al., 2006), prompted us to address the essential function(s) of Wnt5a in hippocampal neurons *in vivo*. Thus, we set out to delete Wnt5a in hippocampal neurons in mice by crossing floxed Wnt5a mice (*Wnt5a*$^{fl/fl}$) with two different transgenic mouse lines, *Nestin-Cre* (Tronche et al., 1999) and *CaMKII□-Cre* (T29-1 line) mice (Tsien et al., 1996a), respectively. The *Nestin* promoter drives Cre expression in all neuronal progenitors starting around day E10 (Tronche et al., 1999). Thus, in *Nestin-Cre;Wnt5a*$^{f/f}$ animals (henceforth called *Nestin-Wnt5a*$^{fl/fl}$ mice), Wnt5a would be expected to be globally deleted in all neurons starting at embryonic stages before the onset of Wnt5a expression in the hippocampus (Figure 3.1). In *CaMKII□-Cre* mice, Cre expression starts at 2.5 weeks after birth and is restricted exclusively to excitatory neurons in the forebrain (Tsien et al., 1996a). Thus, in *CaMKIIa-Cre;Wnt5a*$^{fl/fl}$ mice (referred to as *CaMKII-Wnt5a*$^{fl/fl}$ mice), Wnt5a deletion would occur postnatally well after the onset of Wnt5a expression, and specifically in forebrain excitatory neurons including those in the hippocampus (Figure 3.1).

*Nestin-Wnt5a*$^{fl/fl}$ mice were born in expected Mendelian ratios, were morphologically indistinguishable from their wild-type littermates, were fertile, and survived to adulthood up until the last examined age of one and half years. By *in situ* hybridization, *Wnt5a*

transcript was undetectable in the *Nestin-Wnt5a*$^{fl/fl}$ hippocampus throughout postnatal ages including postnatal day 21 (P21) (Figure 3.2). Quantitative real-time PCR (qPCR) analysis showed that *Wnt5a* mRNA was substantially reduced by 70 ± 6% as early as one week after birth (Figure 3.3), and decreased by 83 ± 3.3% at 1.5 months of age in the *Nestin-Wnt5a*$^{fl/fl}$ hippocampus (Figure 3.4). qPCR analysis also showed that levels of other *Wnt* transcripts were unaltered in the absence of Wnt5a at 1.5 months of age (Figure 3.4), indicating that the loss of Wnt5a does not elicit compensatory changes in the expression of other *Wnt* genes in the hippocampus. In all these and later analyses, litter-mate *Wnt5a*$^{fl/fl}$ mice were used as controls.

We probed overall hippocampal cytoarchitecture and neuronal projections in the *Nestin-Wnt5a*$^{fl/fl}$ mice using immunostaining with antibodies against NeuN, MAP2, and neurofilament to visualize neuronal nuclei, dendrites and axons, respectively. Despite the early and broad deletion of *Wnt5a* in *Nestin-Wnt5a*$^{fl/fl}$ mice, these analyses revealed no obvious differences in gross morphology and projections of the hippocampal layers between mutants and control litter-mates at 1 month of age (Figures 3.5 A-C), when hippocampal neural circuit establishment should be complete (Pokorny and Yamamoto, 1981a, b; Stanfield and Cowan, 1979). To probe neuronal morphology in greater detail, we then examined the dendritic morphology of CA1 hippocampal pyramidal neurons using Golgi impregnation and tracing of entire dendrite arbors of stained neurons under high magnification using Neurolucida software. We found no obvious differences in the reconstructed dendrite arbors between *Nestin-Wnt5a*$^{fl/fl}$ and *Wnt5a*$^{fl/fl}$ animals at 1 month of age (Figure 3.6 A). Quantitative analyses revealed no significant differences in dendrite length and complexity between mutant and control animals at this stage (Figures

3.6 A and C and D). Additionally, Sholl analyses revealed no differences in the apical or basal dendrite complexity between the genotypes (Figures 3.7 A-B). These findings were surprising given the reported functions of Wnt5a in regulating axon and dendrite morphogenesis in cultured hippocampal neurons (Bian et al., 2015; Varela-Nallar et al., 2010a; Zhang et al., 2007).

In stark contrast to the normal appearance of dendrites in 1 month-old *Nestin-Wnt5a*$^{fl/fl}$ mice, we observed striking differences between mutant mice and controls at 6 months (Figure 3.6 B). There was a marked decrease in dendrite length and complexity with impairments observed in both apical and basal dendrite arbors in 6 month old *Nestin-Wnt5a*$^{fl/fl}$ compared to control mice (Figures 3.6 B, C and E; Figures 3.7 C and D). Total dendritic length was reduced by ~50% in mutant mice (Figure 3.6 C). Based on Sholl analyses, it appeared that distal dendrites at distances 50 μm and further from the soma were more severely affected than proximal dendrites (Figure 3.6 E). Notably, in control mice, the total dendritic length remained remarkably stable between 1 and 6 months of age (3932.3 ± 123.2 □m at 1 month *versus* 3943.7 ± 169.3 □m at 6 months) (Figure 3.6 C). Also, dendrite arbors, reported to reach their mature size by P21 (Sfakianos et al., 2007), showed only a modest expansion with age specifically in the distal dendrites in control mice (compare Figures 3.6 D and 3.6 E). In contrast, *Nestin-Wnt5a*$^{fl/fl}$ mice exhibited a pronounced decrease in dendritic length between 1 and 6 months of age (3779.4 ± 124 □m at 1 month *versus* 2115 ± 63.7 □m at 6 months) and dendrite complexity (Figures 3.6 D and 3.6 E). We further quantified densities of dendritic spines, believed to be the sites of the majority of excitatory input in the hippocampus, in Golgi-stained neurons by tracing all the visible spines along the shafts of

several apical dendrites, and observed that the average spine density was comparable between *Nestin-Wnt5a*$^{fl/fl}$ and wild-type animals at from p21 and 1 months. However start at 3 months to 6 months, mutant mice showed a significant reduction in spine densities (41% decrease in 6 months muatnt mice) compared to controls (Figures 3.8 A and B). Despite the profound dendritic shrinkage in adult *Nestin-Wnt5a*$^{fl/fl}$ mice, we did not observe any overt cell loss in these animals even when examined up to 12 months of age (Figures 3.9 A and B). By 1 month of age, dendrite arbor elaboration has been largely completed in rodents and spine densities are at peak numbers in CA1 neurons (Pokorny and Yamamoto, 1981a). Together, these results imply that Wnt5a is dispensable for establishing dendritic arbors and for spine formation in CA1 pyramidal neurons. Rather, it is essential for maintaining dendrite morphology in adult neurons.

Like *Nestin-Wnt5a*$^{fl/fl}$ mice, *CaMKII-Wnt5a*$^{fl/fl}$ mice were born in expected Mendelian ratios, had no gross morphological abnormalities, were fertile, and viable. *In situ* hybridization and qPCR analyses showed that *Wnt5a* deletion was near complete in the hippocampus by 3 months of age (Figures 3.10 A and B). In 3 month-old *CaMKII-Wnt5a*$^{fl/fl}$ mice, we found no alterations in dendrite arbor complexities and spine densities compared to control *Wnt5a*$^{fl/fl}$ litter-mates (Figures 3.11 A, C and Figures 3.12 A,B). However, by 6 months of age, we observed a profound loss of dendritic branches and spines (Figures 3.11 B, D and Figures 3.12 A,B), similar to the phenotypes observed in 6-month *Nestin-Wnt5a*$^{fl/fl}$ mice. These results strengthen the notion that Wnt5a is essential for maintaining dendrite arborization and spine densities in adult CA1 hippocampal pyramidal neurons.

Previous data shown wnt5a signaling have dramatic impact in maintenance of adult hippocampal dendritic structure. Wnts are identified as secrected morphogen during animal development. However, whether wnt5a acts through either autocrine or paracrine pathway to support adult hippocampal pyramidal neurons function and structure maintenance still not clear. To test this, 2 month old adult *Wnt5a*$^{f/f}$ animals were stereotaxic injected with lentivirus carrying GFP or GFP-T2A-CRE. Mice were harvested for morphological analyses 90 days after virus injection (Fig 3.13 A). To obtain neuronal morphology in greater detail, both GFP and GFP-T2A-CRE sparse labelled neurons were scanned through confocol microscope and GFP filled dendritic structures were traced and analyzed with Imaris software (Fig 3.13 B and C). Sholl analysis revealed that there was a marked decrease in dendrite complexity (Fig 3.13 D) in GFP-T2A-CRE labelled neurons compared to GFP labelled neurons. Total dendritic length was significantly reduced by 36 % in GFP-T2A-CRE labelled neurons (Figure 3.13 E). Interestingly, wnt5a deletion in the sparse GFP-T2A-CRE labelled neurons have already caused shrinkage of neuronal morphology, while the surrounding neighbor neurons still have ability to secrete wnt5a. These results suggest that Wnt5a acts through autocrine signaling and is essential for dendritic structure maintenance in adult hippocampal neuron.

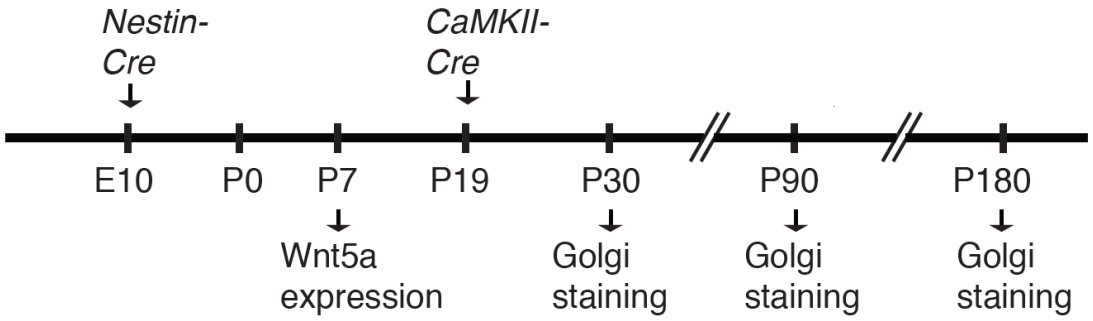



**Figure 3.1: The strategy to utilize *Nestin-CRE* and *CaMKII-CRE* mice lines to address the role of Wnt5a in hippocampus development or adulthood.**

Schematic illustration of the strategy for early- and late-onset deletion of *Wnt5a* using *Nestin-Cre* and *CaMKII-Cre* mice, respectively. Arrows indicate onset of Wnt5a expression in the hippocampus (P7) relative to reported onset of *Nestin-Cre* (E10) and *CaMKII-Cre* expression (P19), as well as when mice were sacrificed for Golgi staining by P30, P90 or P180.

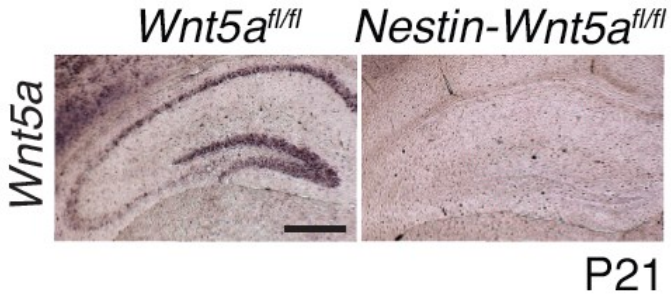



**Figure 3.2: *Wnt5a* transcript is ablated in *Nestin-Wnt5a*$^{fl/fl}$ hippocampus at postnatal day 21.**

*Wnt5a* transcript is undetectable in the *Nestin-Wnt5a*$^{fl/fl}$ hippocampus compared to the control tissue at postnatal day 21 (P21). Scale bar: 500 □m.

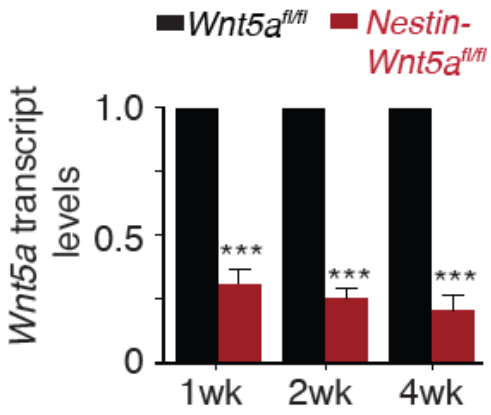



**Figure 3.3: *Wnt5a* transcript is ablated as early as 1 week old *Nestin-Wnt5a*$^{fl/fl}$ mice.**

qPCR analysis shows substantial depletion of *Wnt5a* transcripts in the postnatal *Nestin-Wnt5a*$^{fl/fl}$ hippocampus. Results are means ± SEM from n=7 mice per genotype for 1 and 2 weeks, and n=8 mice per genotype for 4 weeks, ***p<0.001, unpaired two-tailed *t* test.

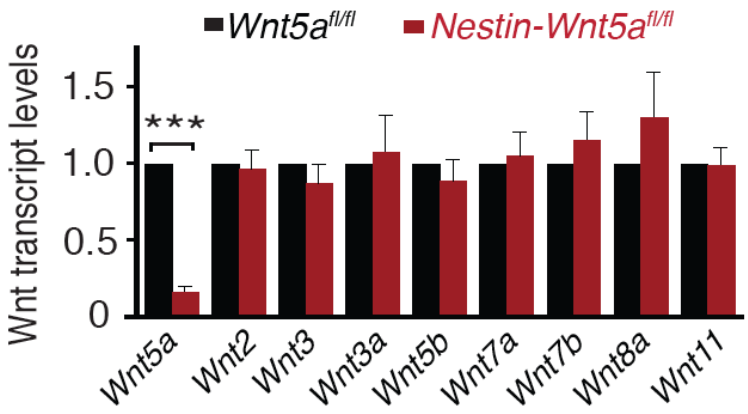



**Figure 3.4: Wnt5a ablation does not alter expressions of other Wnts in hippocampus.**

*Wnt5a* transcript levels are substantially decreased in 1.5-month old *Nestin-Wnt5a*$^{fl/fl}$ mice as determined by qPCR analysis, whereas other hippocampal *Wnts* are unaltered. Results are mean ± SEM from 6 independent experiments. ***p<0.001, unpaired, two-tailed *t* test.

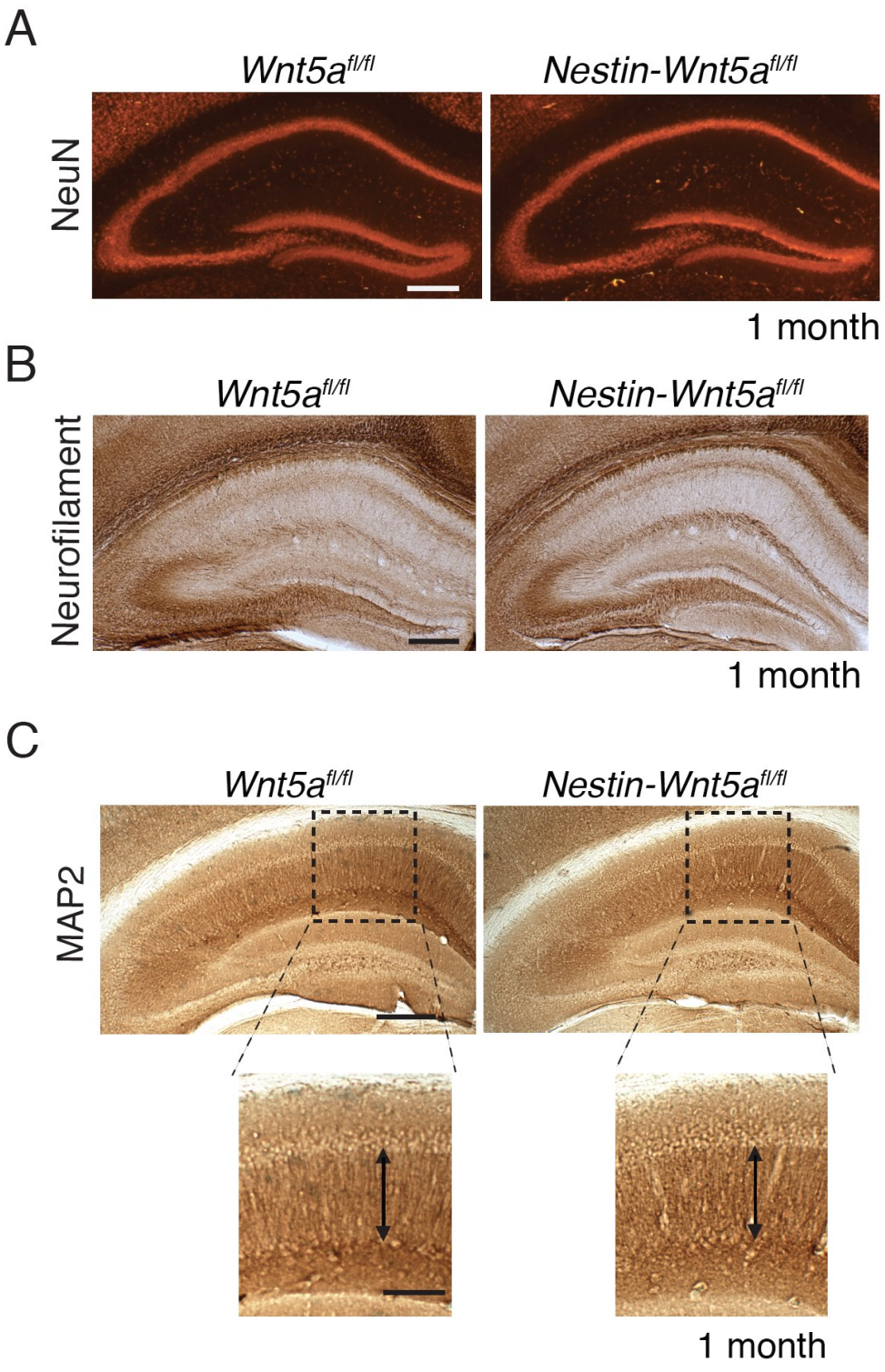



**Figure 3.5: Gross hippocampal morphologies in 1 month-old *Nestin-Wnt5a* mice.**

(**A**) Hippocampal cytoarchitecture is grossly normal in 1 month old *Nestin-Wnt5a*$^{fl/fl}$ mice as assessed by NeuN immunostaining. Scale bar: 400 □m. **(B)** Neurofilament immunohistochemistry shows that axonal projections are unaltered in *Nestin-Wnt5a*$^{fl/fl}$ hippocampus. Scale bar: 400 □m. **(C)** MAP2 staining shows that CA1 dendrite layers are comparable between *Nestin-Wnt5a*$^{fl/fl}$ and control litter-mates at 1 month. Lower panels indicate higher magnification images of the boxed regions. Upper panel scale bar: 400 □m; lower panel scale bar: 200 □m.

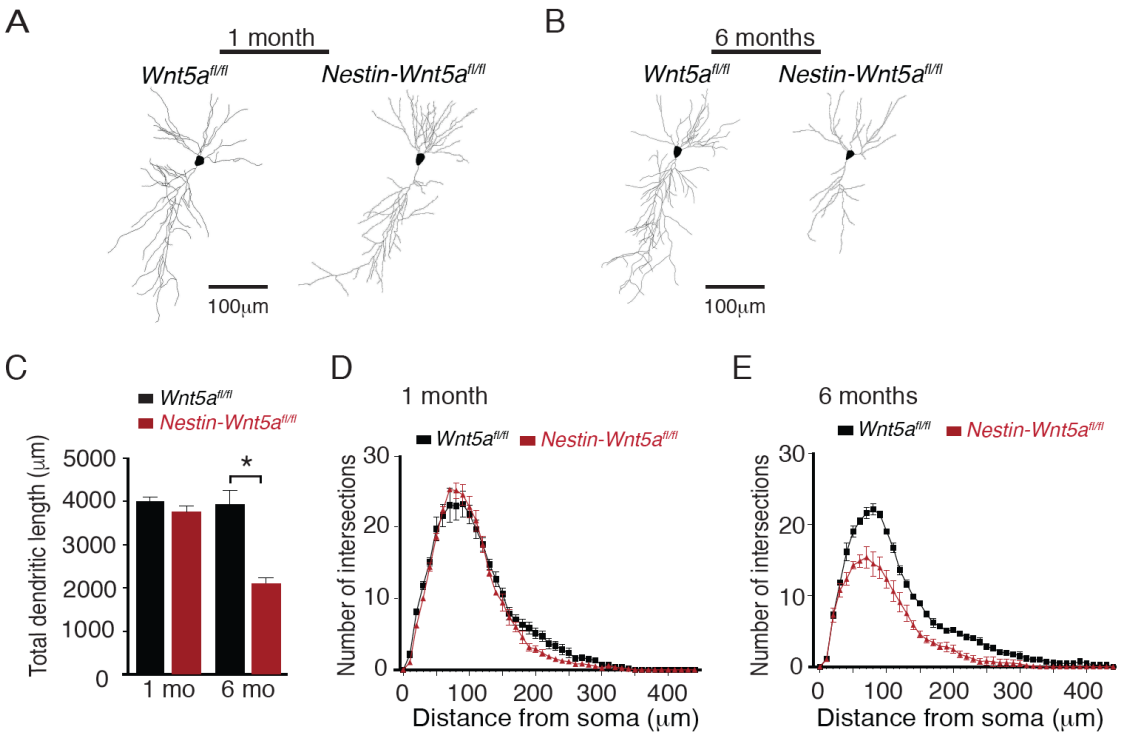



**Figure 3.6: Total dendritic length and dendritic complexity are normal in 1 month old *Nestin-Wnt5a$^{fl/fl}$* mice but have deficit in 6 month old *Nestin-Wnt5a$^{fl/fl}$* mice.**

**(A, B)** Representative reconstructions of Golgi-stained CA1 pyramidal neurons from control and mutant mice at 1 and 6 months. Dendritic arbors are normal in 1 month old *Nestin-Wnt5a$^{fl/fl}$* mice, but significantly reduced at 6 months compared to controls. Scale bar: 100 □m. **(C)** Total dendritic lengths are unaltered in 1 month old *Nestin-Wnt5a$^{fl/fl}$* mice but significantly decreased by 6 months, compared to litter-mate *Wnt5a$^{fl/fl}$* controls. Results are mean ± SEM from 5 neurons traced per animal and a total of 4 mice per genotype. *p<0.05, unpaired two-tailed *t* test. **(D, E)** Sholl analyses show decreased dendrite branching complexity in CA1 pyramidal neurons in 6 month, but not 1 month-old *Nestin-Wnt5a$^{fl/fl}$* mice.

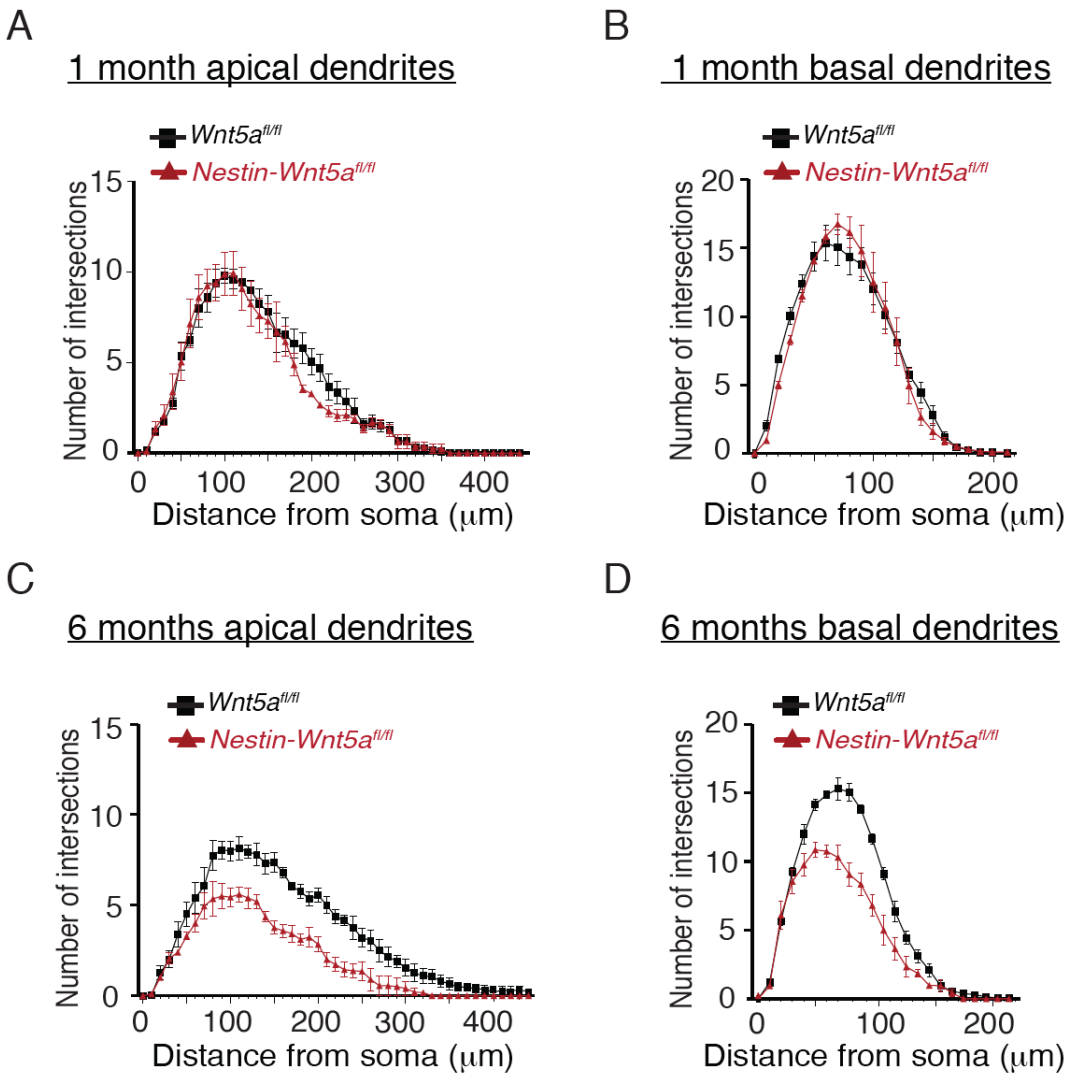



**Figure 3.7: Both apical and basal dendritic complexity are normal in 1 month old *Nestin-Wnt5a*$^{fl/fl}$ mice but show deficit in 6 month old *Nestin-Wnt5a*$^{fl/fl}$ mice**

**(A-D)** Sholl analyses show that apical and basal dendrite complexities of CA1 pyramidal neurons are unaltered in *Nestin-Wnt5a*$^{fl/fl}$ mice at 1 month, but markedly reduced in 6 month old mutants compared to controls

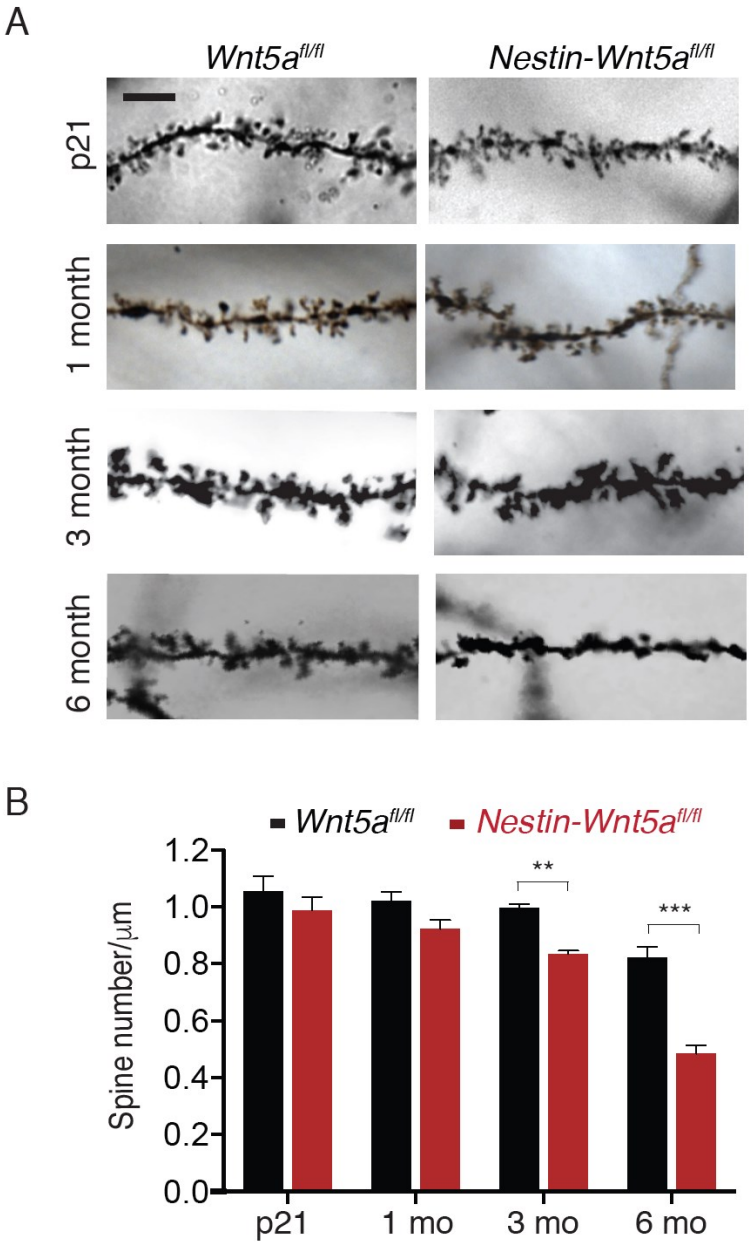



**Figure 3.8: Spine densities are normal in the developmental stage of *Nestin-Wnt5a*$^{fl/fl}$ mice but start to reduce in 3 and 6 month old *Nestin-Wnt5a*$^{fl/fl}$ in hippocampal CA1 pyramidal neurons.**

**(A, B)** Representative Golgi-stained segments of CA1 apical dendrites from *Nestin-Wnt5a*$^{fl/fl}$ mice and littermate controls at p21, 1, 3 and 6 months of age. Spine densities

are normal at p21 after birth but gradually reduced at from 3 months and 6 months old *Nestin-Wnt5a*$^{fl/fl}$ mice compared to *Wnt5a*$^{fl/fl}$ littermates. Scale bar: 5□m. Results are mean ± SEM from n=8 *Nestin-Wnt5a*$^{fl/fl}$ mice and n=5 *Wnt5a*$^{fl/fl}$ mice at p21; 5 mice per genotype at 1 month; 4 mice per genotype at 3 months; n=4 *Nestin-Wnt5a*$^{fl/fl}$ mice and n=6 *Wnt5a*$^{fl/fl}$ mice at 6 months. *$p<0.05$, unpaired two-tailed *t* test.

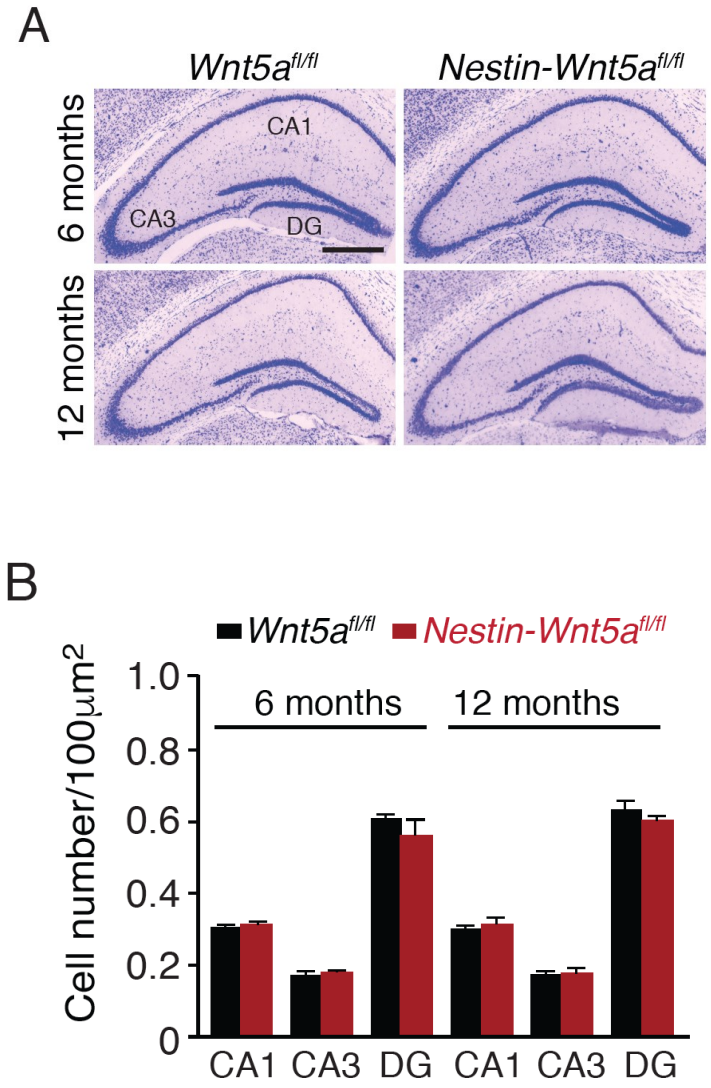



**Figure 3.9: No cell loss in 12 month *Nestin-Wnt5a*$^{fl/fl}$ mice.**

(**A, B**) Nissl staining and cell counts in CA1, CA3, and dentate gyrus layers reveal similar cell densities between *Nestin-Wnt5a*$^{fl/fl}$ and control mice at 6 and 12 months. Scale bar: 500 □m. Results are mean ± SEM from 3 mice per genotype.

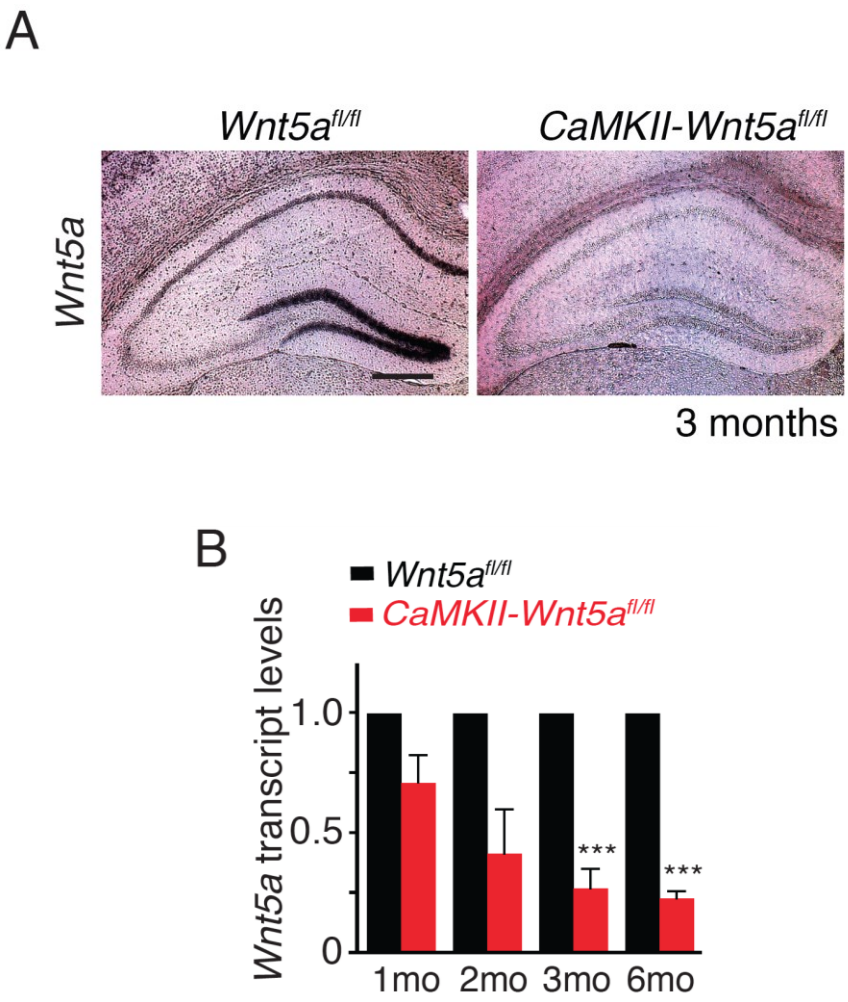



**Figure 3.10: *Wnt5a* transcript is ablated in 3 month old *CaMKII-Wnt5a$^{fl/fl}$* mice.**

**(A)** *Wnt5a* transcript is ablated by 3 months in *CaMKII-Wnt5a$^{fl/fl}$* mice as shown by *in situ* hybridization. Scale bar: 400 □m. **(B)** *Wnt5a* transcript levels are substantially decreased in 3 month old *CaMKII-Wnt5a$^{fl/fl}$* mice as determined by qPCR analysis. Results are means ± SEM from n=4 mice per genotype for 1 and 2 months, and n=5 mice per genotype for 3 and 6 months**,** ***p<0.001, unpaired two-tailed *t* test.

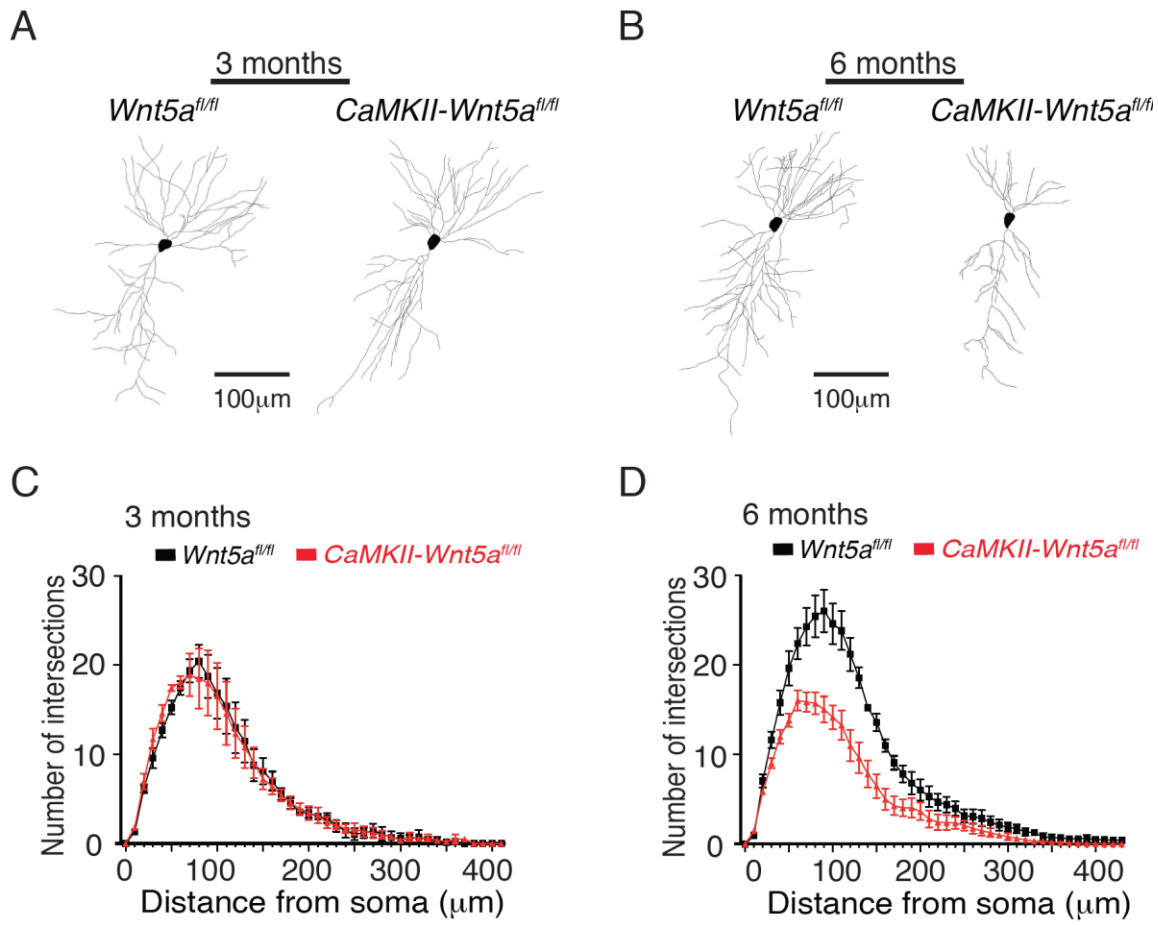



**Figure 3.11: Dendritic complexity are normal in 3 month old *CaMKII-Wnt5a$^{fl/fl}$* mice but have deficit in 6 month old *CaMKII-Wnt5a$^{fl/fl}$* mice.**

(**A, B**) Dendritic arbors are normal in 3-month old *CaMKII-Wnt5a$^{fl/fl}$* mice but reduced in size at 6 months of age. Scale bar 100 □m. (**C, D**) Sholl analyses show pronounced shrinkage of dendrite arbors of CA1 pyramidal neurons at 6 but not 3 months of age in *CaMKII-Wnt5a$^{fl/fl}$* mice, compared to litter-mate controls.

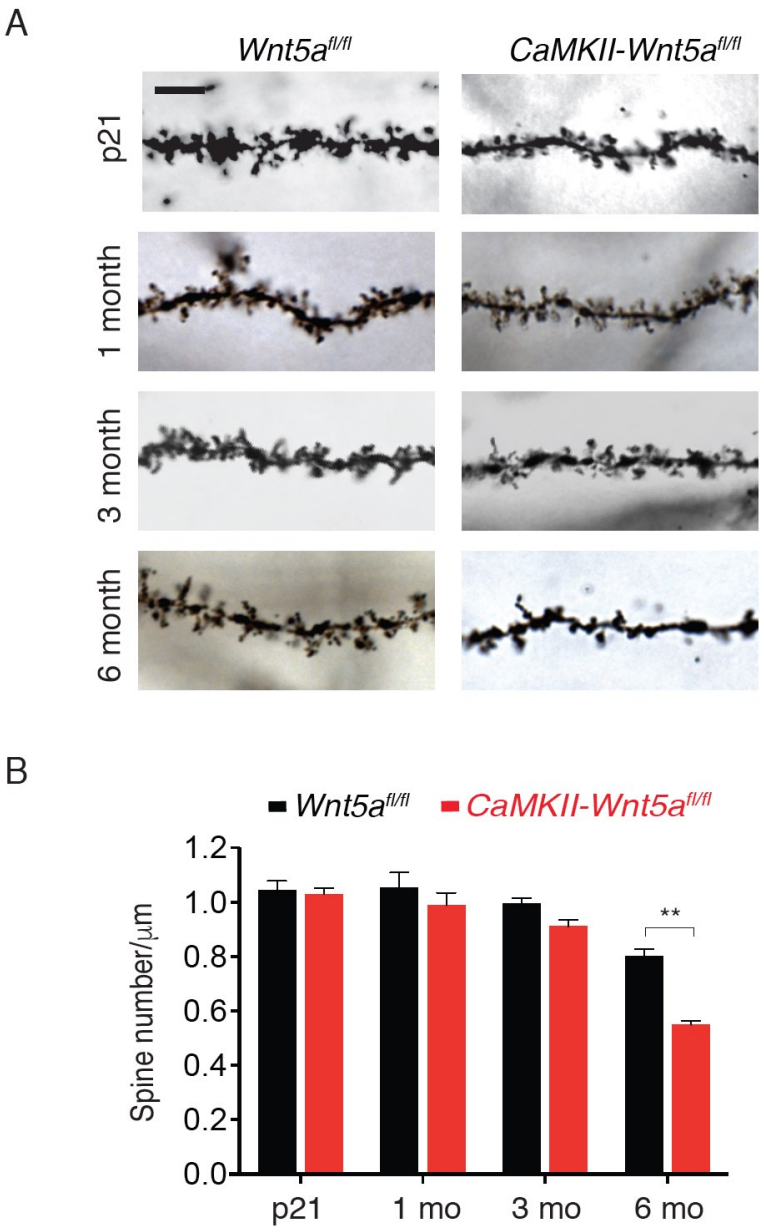



**Figure 3.12: Spine densities are normal in early stage of *CaMKII-Wnt5a$^{fl/fl}$* mice but have deficit in 6 month old *CaMKII-Wnt5a$^{fl/fl}$* in hippocampal CA1 pyramidal neurons.**

**(A, B)** Spine densities are significantly reduced by 6 months, but normal at 3 months in *CaMKII-Wnt5a$^{fl/fl}$* mice. Scale bar: 5□m. Results are means ± SEM from 3 mice per

genotype at p21; n=8 for *CaMKII-Wnt5a*$^{fl/fl}$ mice and n=5 for *Wnt5a*$^{fl/fl}$ mice at 1 month; 5 mice per genotype at 3 months, and n=7 for *CaMKII-Wnt5a*$^{fl/fl}$ mice and n=5 for *Wnt5a*$^{fl/fl}$ mice at 6 months. **p<0.01, unpaired two-tailed *t* test.

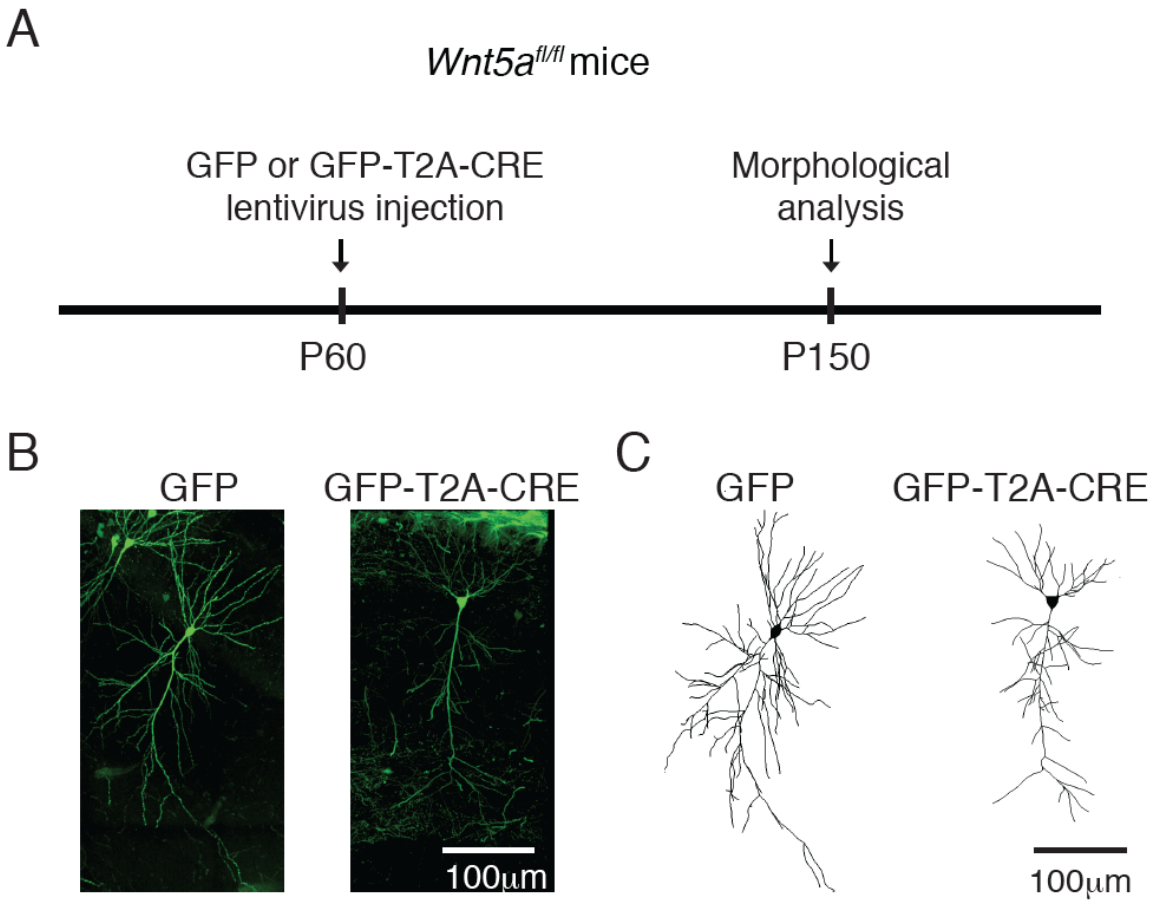

A
Wnt5a fl/fl mice
GFP or GFP-T2A-CRE
lentivirus injection
Morphological
analysis
P60
P150
B
GFP
GFP-T2A-CRE
100μm
C
GFP
GFP-T2A-CRE
100μm
D
Number of intersections
30
20
10
0
0
100
200
300
400
Distance from soma (μm)
GFP
GFP-T2A-CRE
E
GFP
GFP-T2A-CRE
Total dendritic length (μm)
6000
5000
4000
3000
2000
1000
0
***

**Figure 3.13: Wnt5a autocrine signaling is essential for dendritic structure maintenance in the adult mice.**

**(A)** Schematic illustration of the strategy for lentivirus deletion in adult *Wnt5a*$^{fl/fl}$ mice. Mice infect with either GFP or GFP-T2A-CRE lentivirus at 2 month and analyze pyramidal dendritic structure at 5 month. Arrows indicate time point of lentivirus deliver and mice were sacrificed for Imaris dendritic structure tracing at P150. **(B)** Images with GFP filled sparse labelled wild type neuron (left), and sparse labelled *wnt5a* deleted neuron (right). **(C)** Imaris 3 dimension neuronal tracing shown in (B). (**D)** Sholl analyses show pronounced shrinkage of dendrite arbors of CA1 pyramidal neurons at GFP-T2A-CRE infected *Wnt5a*$^{fl/fl}$ neuron, compared to GFP infected *Wnt5a*$^{fl/fl}$ neuron. (**E)** Total dendritic lengths are significantly decreased in GFP-T2A-CRE infected *Wnt5a*$^{fl/fl}$ neuron, compared to GFP infected *Wnt5a*$^{fl/fl}$ neuron as control. Results are mean ± SEM from 10 GFP infected *Wnt5a*$^{fl/fl}$ neurons and 14 GFP-T2A-CRE infected *Wnt5a*$^{fl/fl}$ neurons. ***$p<0.001$, unpaired two-tailed *t* test.

## Discussion

In order to dissect out Wnt5a functions in the central nervous system, we crossed *Wnt5a*$^{fl/fl}$ mice with two different Cre lines. *Nestin-Cre* mice would allow us to delete Wnt5a in embryonic day 10 which is much earlier than Wnt5a expression in the brain. With *Nestin-Wnt5a*$^{fl/fl}$ mice, we can specific address Wnt5a function in hippocampal neurons during animal early development. Cre reconbinase is expressed in postnatal day 19 in *CaMKII-Wnt5a*$^{fl/fl}$ mice which Wnt5a be deleted in early adulthood. Analysing *CaMKII-Wnt5a*$^{fl/fl}$ mice would help us to understand more of Wnt5a functions in hippocampal pyramidal neurons specifically in the adulthood.

We found that the early deletion of Wnt5a in neurons did not elicit any structural abnormalities in spine density and dendritic complexity in CA1 pyramidal neurons in 1 month old *Nestin-Wnt5a*$^{fl/fl}$ mice. These results suggest that neuronal Wnt5a is dispensable for the establishment or maturation of hippocampal connectivity *in vivo*. These findings were surprising in the context of reported developmental functions for Wnt5a in cultured hippocampal neurons, and in embryonic processes in other brain regions (Bian et al., 2015; Varela-Nallar et al., 2010a; Zhang et al., 2007). In hippocampal neurons, several signaling pathways have been shown to influence dendrite morphogenesis, maturation and stability *in vitro* and *in vivo* (Emoto, 2012; Koleske, 2013; Valnegri et al., 2015). Thus, in the absence of Wnt5a, other signaling mechanisms including other Wnt molecules (Rosso et al., 2005; Wayman et al., 2006), could provide trophic support to hippocampal CA1 dendrite arbors and spines formation for the first months of life in mice. Alternatively, Wnt5a derived from other cell population may also support hippocampal formation in the absence of neuron-derived Wnt5a. However, the

profound defects in hippocampal dendritic complexity and spine density in older *Nestin-Wnt5a*$^{fl/fl}$ and *CaMKII-Wnt5a*$^{fl/fl}$ mice suggest that these mechanisms are unable to compensate for Wnt5a loss at later stages of life. Rather, our results suggest that Wnt5a, likely derived from CA1 pyramidal neurons themselves, is necessary for sustaining dendritic architecture in the adult hippocampus. Based on these findings, we conclude that Wnt5a is essential for the maintenance of adult CA1 hippocampal neurons and is relevant to the structural bases of hippocampus-dependent behaviors.

## Methods

### Animals

All procedures relating to animal care and treatment conformed to institutional and NIH guidelines. Animals were housed in a standard 12:12 light-dark cycle. The generation of *Wnt5a*$^{fl/fl}$ mice has been previously described (Ryu et al., 2013). *Wnt5a*$^{fl/fl}$ mice were backcrossed to *C57BL/6* background for at least 7 generations and maintained on a *C57BL/6* background. *Nestin-Cre* and *TOPGAL* mice were obtained from Jackson Laboratory and *CaMKII-Cre* mice (T29-1 line) were a generous gift from Dr. Nicholas Gaiano. Sprague Dawley rats were purchased from Charles River. Hippocampal neuron cultures were established from embryonic day 18 (E18) rat pups as previously described (Araki et al., 2015)

### *In situ* hybridization

*In situ* hybridization was performed using a digoxigenin labeled probe spanning a 572 bp region within *Wnt5a* exon 2. Mouse brains of various ages were fresh frozen in OCT (Tissue-Tek) and serially sectioned (20μm) using a cryostat. Sections were post-fixed in 4% paraformaldehyde (PFA), washed in PBS and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine with 0.9% NaCl. After hybridization with the labeled RNA probe (2 □g/ml) at 68°C overnight, sections were washed with 0.2X SSC buffer at 65°C, blocked with TBS containing 1% normal goat serum and then incubated with alkaline phosphatase-labeled anti-DIG antibody (1:5000; Roche) overnight at 4°C. The alkaline phosphatase reaction was visualized with NBT/BCIP, rinsed in PBS, fixed in 4% PFA and mounted in AquaMount (EMD Chemicals).

**KCl-mediated depolarization or bicuculline treatment of cultured hippocampal neurons**

Hippocampal neurons were harvested from E18 Sprague-Dawley rats and grown in poly-L-lysine coated 12 well plates. Cells were maintained in plates with Neurobasal media supplemented with B27, penicillin/streptomycin (1U/ml), and GlutaMax. Cultured hippocampal neurons were treated with TTX (1 μM/ml) for 48 hr on d.i.v 18 to silence neuronal activity, then TTX was washed away using several washes with Neurobasal culture media, and neurons were then stimulated with either KCl (20mM) or Bicuculline (40 μM/ml) for different times.

**Electro-convulsive shock (ECS)**

Electro-convulsive shock was administered in 8 week-old male *C57BL/6* mice as previously described (Ramanan et al., 2005). Following attachment of saline-soaked ear clips to the mice, 15mA electric pulses (100 Hz, 0.4 ms/ pulse) were delivered through mouse brains for 1 sec using the Ugo Basile ECT unit, Model 57800. Hippocampal tissues were harvested at 1 hr or 4 hr after ECS treatment, and subjected to qPCR or immunoblotting analyses for Wnt5a and Arc. Total protein concentrations were estimated using the BCA assay.

**Real time-PCR analyses**

Total RNA was prepared from dissected hippocampal tissues using Trizol-chloroform extraction (ThermoFisher; 10296-010). RNA was then reverse transcribed using a RETROscript Reverse Transcription kit (ThermoFisher; AM1710). Real-time qPCR was

performed using a Maxima SYBR Green/Rox Q-PCR Master Mix (ThermoFisher; K0221), in 7300 Real time PCR System (Applied Biosystems). The following primers were used for the analyses; *Ar*c-F: 5'-TGAGACCAATTCCACTGATG-3' and *Arc*-R: 5'-CTCCAGGGTCTCCCTAGTCC-3'; *Wnt5a*-F:5'-CTCGGGTGGCGACTTCCTCTCCG-3' and *Wnt5a*-R:5'-CTATAACAACCTGGGCGAAGGAG-3'; *Wnt2*-F:5'-GTAGATGC CAAGGAGAGGAAAG-3' and *Wnt2*-R:5'-CCACTCACACCATGACACTT-3'; *Wnt3*-F:5'-TGGACCACATGCACCTAAAG-3' and *Wnt3*-R:5'-CGTACTTGTCCTTGAGGA AGTC-3'; *Wnt3a*-F:5'-GCAGCTGTGAAGTGAAGAC-3' and *Wnt3a*-R:5'-GGTGTT TCTCTACCACCATCTC-3'; *Wnt5b*-F:5'-GAGAGCGTGAGAAGAACTTTG-3' and *Wnt5b*-R: 5'-GCGACATCAGCCATCTTATAC-3'; *Wnt7a*-F: 5'-GCCTTCACCTAT GCGATTATC-3' and *Wnt7a*-R: 5'- GGTACTGGCCTTGCTTCTC-3'; *Wnt7b*-F: 5'-GCATGAAGCTGGAATGTAAGTG-3' and *Wnt7b*-R: 5'-TGCGTTGTACTTCTCCTT GAG-3'; *Wnt8a*-F: 5'-TGGGAACGGTGGAATTGTC-3' and *Wnt8a*-R: 5'-GCGGA TGGCATGAATGAAG-3'; *Wnt11*-F: 5'-CCTGGAAACGAAGTGTAAATGC-3' and *Wnt11*-R: 5'-TGACAGGTAGCGGGTCTTG-3'; *GluN1*-F: 5'-CCAGATGTCCACCA GACTAAAG-3' and *GluN1*-R: 5'-CATTGACTGTGAACTCCTCTTTG-3'; *GluN2a*-F: 5'-CTGTGTGGCCAAGGTATAAG-3' and *GluN2a*-R: 5'-TCAGTCAGTGGGTC TATGTC-3'; *GluN2b*-F: 5'-ATGAGGAACCAGGCTACATC-3' and *GluN2b*-R: 5'-GGTCACCAGGTAAAGGTCATAG-3'; *Axin2*-F: 5'-GAGAGTGAGCGGCAG AGC-3' and *Axin2*-R: 5'-CGGCTGACTCGTTCTCCTG-3'; *GAPDH*-F: 5'-CCTGCACCACC AACTGCTTA-3' and *GAPDH*-R: 5'-CCACGATGCCAAAGTTGTCA-3'. Each sample was analyzed in triplicate reactions. Data were analysed using the $\Delta\Delta C_t$ method,

normalizing each sample to the internal control, and relative messenger RNA was determined as the percentage of the maximum value observed in the experiment.

**Immunohistochemical analyses**

Mouse brains were fixed in 4% paraformaldehyde (PFA) at 4°C overnight, cryoprotected in 30% sucrose in PBS, frozen in OCT (Tissue-Tek) with dry ice and serially sectioned (40 □m). For immunohistochemistry with diaminobenzidine (DAB), sections were first incubated with 10% methanol +3% $H_2O_2$ for 20 min to quench peroxidase activity and then washed in TBS, permeabilized in TBS containing 0.4% Triton X-100, and blocked using 10% goat serum + 3% BSA for 2 hrs. Sections were incubated in the following primary antibodies overnight: chicken anti-neurofilament (EMD Millipore; AB5539, 1:500) or mouse anti-MAP2 (Sigma Aldrich; M9942, 1:1500). Following TBS washes, sections were incubated with either chicken or mouse anti-HRP secondary antibodies (GE Healthcare, 1:500) for 1 hr. Sections were then washed in TBS, incubated with diaminobenzidine (DAB) for 2-10 min at room temperature, followed by washes in TBS and then mounted in VectaShield (Vector Laboratories).

For immunofluorescence, sections were washed in TBS, permeabilized in TBS containing 0.4% Triton X-100, and blocked using 10% goat serum + 3% BSA for 2 hrs. Sections were then incubated in primary antibodies overnight. Following TBS washes, sections were incubated with Alexa-Fluor conjugated secondary antibodies (ThermoFisher, 1:500) for 1 hr. Sections were washed in TBS and then mounted in VectaShield (Vector Laboratories).

**Golgi staining**

Golgi staining was performed with FD Rapid Golgistain kit on mouse brains that were fresh frozen in OCT and serially sectioned (200μm) using a cryostat. Dendritic arbors were analyzed as described previously (Xie et al., 2010). Briefly, Golgi-impregnated pyramidal neurons in the CA1 region were traced using Neurolucida software (MicroBrightField) under a Nikon Eclipse E800 microscope, equipped with a motorized stage. Analyses were done by an investigator blinded to the genotype. All of the analyzed neurons were well stained, isolated and had intact dendritic arbors. Dendritic length of each traced neuron was calculated using NeuroExplorer software (MicroBrightField). 5 neurons were traced per animal, and the average used as the value for each mouse during statistical analyses.

To measure spine density, we took images of distal apical dendrites (>100 μm away from the soma) of hippocampal CA1 pramidal neurons at 63x magnification using a Zeiss Axiovision microscope with a AxioCam HRC digital camera. The position of each dendritic spine along these dendrite segments was assessed manually and counted using ImageJ software. Spine density for each animal was obtained from at least 15 dendrites per mouse with a total length of 2000~3000 μm traced for statistical analys

**Statistical analyses**

All Student's *t* tests were performed using two-tailed, unpaired, and a confidence interval of 95%. One-way or two-way ANOVA analyses were performed when more than two groups were compared. Statistical analyses were based on at least 3 independent

experiments, and described in the figure legends. All error bars represent the standard error of the mean (s.e.m).

# Chapter 4: Wnt5a functions in synaptic plasticity and spatial learning and memory

## Introduction

The human brain is comprised of billions of neurons that form complex circuitry and enable us to interact with the outer environment, experience with new thing and storage memory. Synapses are connections between neurons and are thus critical for information propagation throughout the brain network. With the flexibility of neurons to respond to the changing environment, we are able to learn novel things and form memory. Even before birth, synaptic plasticity has played important roles during early development. In the visual system, the neuronal circuitry is further shaped in response to sensory information from light after eye opening. The synaptic connectivity and strength are constantly modified based on the environmental stimulation throughout our lifetime (Clarke et al., 2012; Feldmeyer and Radnikow, 2009; Kerchner and Nicoll, 2008; Sanes and Kotak, 2011). From the infant to teenage stages, our brains need to learn and process enormous new information, for example family interaction, language communication, learning skill for living, and absorbing culture for life in a short period of time. These stages require high levels of synaptic plasticity to accomplish novel tasks. In the adult stage although most experiences have learned, processed and memorized from the previous stages and the need for synaptic plasticity becomes relative less, the plasticity is still required for new stimulations to continuous modification and adaptation in this stage (Feldmeyer and Radnikow, 2009).

Spines are located on the surface of dendrite and each spine may contain zero to a few biochemical and electrical synapses to propagate information and modify synaptic plasticity. There are many factors that can regulate post-synaptic responses during the changes in synaptic plasticity. Within the spines, complex molecular machinery

cooperatively regulates the strength of synaptic transmission and the structure of synapses between neurons. Glutamate receptors on the post-synaptic membrane convert chemical signals into electrical signals and thus are one of the key players in controlling synaptic transmission and plasticity (Anggono and Huganir, 2012; Bassani et al., 2013) . Changes in the number and conductance of glutamate receptors on the synapse membrane can either potentiate or depress synaptic strength (Malinow and Malenka, 2002). Additionally, signaling cascades following glutamate receptor activation at the post-synapses can further modulate synaptic transmission and plasticity. In response to synaptic activation, many kinases and phosphatases work as critical hinges to mediate signaling that can impact not only the synaptic function but also gene transcription.

Synaptic plasticity, long-term potentiation (LTP) and long-term depression (LTD) of synaptic strength are often expressed post-synaptically by the insertion or removal of the glutamate receptor-AMPA receptors (AMPAR)(Bassani et al., 2013; Malinow and Malenka, 2002; Shepherd and Huganir, 2007). Excitatory synapses contain AMPAR to transmit fast synaptic signals and NMDA-type receptors (NMDAR) to trigger long-term changes in synaptic transmission in LTP and LTD (Malinow and Malenka, 2002). During the past decades, many studies on LTP have focused on the signaling mediated by CaMKII (Lisman et al., 2012) and molecular mechanism underlying AMPAR trafficking regulation (Anggono and Huganir, 2012; Kessels and Malinow, 2009; Nicoll and Roche, 2013). Many evidence have shown that CaMKII activation triggered by calcium entry through the NMDAR is both necessary and sufficient for LTP activation. Interestingly, elevated calcium in the spine induces not only CaMKII activity-dependent translation but also translocation of itself into post-synaptic density (PSD) region, which promotes

CaMKII to interact with the synaptic GluN2B (subunit of the NMDAR). GluN2B therefore functions ideally as a CaMKII sequester to remain CaMKII stay active with local calcium elevation at synapses. The active CaMKII is able to phosphorylate other molecules locally in the PSD region. After the initial activation by calcium, CaMKII is thought to auto-phosphorylated and transformed itself into a calcium-independent stage, which allows it to serve as a constitutively active enzyme and function as a ‘‘memory molecule’’ (Lee et al., 2009; Lisman et al., 2012). However, recent two-photon lifetime imaging has shown that the CaMKII activation is only transient and its activity returns to baseline within a few minutes during the activation of LTP (Lee et al., 2009). This evidence indicates that other CaMKII downstream molecules might relay signaling for the persistence of LTP. One of the major substrates downstream of CaMKII is the GluA1 subunit of the AMPAR (Barria et al., 1997; Mammen et al., 1997). CaMKII phosphorylation of GluA1 allows GluA1-containing AMPAR to traffic into synapses and increase the channel conductance (Lu and Roche, 2012; Shepherd and Huganir, 2007)for pS579 ). In addition, CaMKII also phosphorylates many other proteins located in the PSD region, such as PSD-95, SynGAP, and the GluN2B subunit of the NMDAR (Dosemeci and Jaffe, 2010; Yoshimura et al., 2002). Recent study also showed the CaMKII-triggered, local persistent activation of Ras and Rho GTPases (RhoA and Cdc42), which are well established small GTPases important for both spine morphologies and synaptic plasticity (Murakoshi et al., 2011).

**AMPAR Phosphorylation and Synaptic Plasticity**

As early as late 1980s, CaMKII protein kinase activity has been shown to be

sufficient and required for the induction of LTP, suggesting that protein phosphorylation and dephosphorylation might be critical for LTP, LTD and other forms of synaptic plasticity (Malenka et al., 1989; Malinow et al., 1989; Wyllie and Nicoll, 1994). AMPAR consist of four homologous subunits (GluA1-4) that form heteromeric and tetrameric complexes (Traynelis et al., 2010). The major forms of AMPAR in the hippocampus include GluA1/2 and GluA2/3 heteromers and GluA1 homomers (Lu et al., 2009; Wenthold et al., 1996). Now it has been reported that several protein kinases including CaMKII, PKA, PKC, PKG, FYN, and JNK can phosphorylate GluA1-4 subunits on serine, threonine, and tyrosine residues at c-terminal tails (Lu and Roche, 2012; Shepherd and Huganir, 2007). Recent evidence showed that the CaMKII, PKA, and PKC sites on GluA1 tail and the major PKC site on GluA2 tail have major impacts on LTP by regulating AMPARs trafficking and conductance. Phosphorylation of GluA1 by CaMKII is known to regulate AMPAR channel conductance and trafficking (Lee, 2006; Lu and Roche, 2012; Shepherd and Huganir, 2007). The strongest evidence comes from experiments using knockin mice with the GluA1, CaMKII and PKA phosphorylation sites mutations to studty the role of GluA1 phosphorylation in LTP and LTD. GluA1 phospho-mutant mice show deficits in LTP and LTD and have memory defects in spatial learning tasks. These evidence indicate that phosphorylation of GluR1 is critical for synaptic plasticity and the retention of memories (Lee et al., 2003). Interestingly, knockin mice with mutations that mimic the CaMKII and PKA phosphorylation sites on GluA1 showed a lower threshold for LTP induction. Finally, a study using a knockin mouse with the PKC phosphorylation mutant on serine 880 of the GluA2 subunit is eliminated cerebellar LTD in Purkinje neurons (Linden, 2012).

Wnts have been linked to the assembly of structural components in both of pre-synaptic and post-synaptic compartments. In the cerebellum granular cells (GC), Wnt7a functions as a synaptogenic factor that can induce pre-synaptic differentiation in the mossy fiber (Hall et al., 2000). Wnt7a also induces clustering of synapsin I, synaptophysin, synaptotagmin and SV-2 which are important for recycling and controlling exocytosis of synaptic vesicles and in turn regulating synaptic transmission (Ahmad-Annuar et al., 2006; Cerpa et al., 2008; Hall et al., 2000). Moreover, Wnt7a mutant mice shown deficient in synaptic maturation (Ahmad-Annuar et al., 2006). Besides, Wnt3a and Wnt7b, the other cannonical wnt member, also increase the number of pre-synaptic puncta (Cerpa et al., 2008; Davis et al., 2008). Altogether these evidences suggest that canonical Wnt pathway are mostly involved in the pre-synaptic structure assembly and facilitate the nerunoal transmitter release from pre-synaptic terminal.

For the non-canonical Wnt funcion, bath application of Wnt5a in primary cultured hippocampal neurons has been shown to up-regulates excitatory synaptic currents through NMDAR activation, and therefore facilitating the induction of LTP (Cerpa et al., 2011). In addition, Wnt5a is reported to promote dendritic spine formation and increase spine size as well as spine density in cultured hippocampal neurons. Interestingly, Wnt5a also induces local calcium concentration in the synaptic puncta of cultured hippocampal neurons, suggesting that Wnt5a may have the ability to activate downstream Wnt/$Ca^{2+}$ signaling cascade by phosphorylation of CaMKII (Farias et al., 2009; Varela-Nallar et al., 2010a). Together, these *in vitro* results showed a requirement of non-canonical Wnt5a signaling in synaptic transmission and morphology.

Interestingly, *in vivo* activation of Wnt signalings in adult mice by infusion adult mice with either WASP-1, an activator of Wnt/β-catenin pathway or FOXY-5, an activator of both Wnt/JNK and Wnt/$Ca^{2+}$ signaling, enhances their learning and memory performance in the MWM spatial learning and memory test as well as the novel object recognition task (Beaumont et al., 2007; Safholm et al., 2008; Vargas et al., 2014). These findings indicate that both canonical and non-canonical Wnt signaling pathways can enhances animal cognitive behaviors. With newly generated conditional Wnt5a mice, we aim to address the physiology roles of Wnt5a in the synaptic plasticity and behaviors in adult animals with loss of Wnt5a in the hippocampus.

## Result

### Wnt5a is essential for hippocampal synaptic plasticity

Hippocampal neurons exhibit prominent synaptic plasticity in which activity-dependent modulation of the strength of synaptic connections underlies learning and memory (Neves et al., 2008). Given the decreased levels of phosphorylation of CaMKII as well as the down-regulation of GluN1 in hippocampal neurons with Wnt5a deletion, we addressed the *in vivo* requirement for Wnt5a in hippocampal synaptic transmission and plasticity. We analyzed *CaMKII-Wnt5a*$^{fl/fl}$ mice at 3 months of age a time when CA1 neuronal morphology is still intact (see Figures 3.11 A, C and Figures 3.12 A, B). We evaluated basal synaptic transmission by recording field excitatory postsynaptic potentials (fEPSPs) in CA1 neurons evoked by stimulation of Schaffer collaterals in acute hippocampal slices. Input-output relationships, generated by plotting the slope of the fEPSPs *versus* the amplitude of the presynaptic fiber volley at various stimulation intensities, were essentially identical between 3-month *CaMKII-Wnt5a*$^{fl/fl}$ and control mice (Figures 4.1 A, B), indicating that basal synaptic strength is not altered with Wnt5a loss at this age. By 6 months of age when *CaMKII-Wnt5a*$^{fl/fl}$ mice have profound dendritic abnormalities, however, basal synaptic transmission was impaired as the slope of input-output curve was significantly decreased in mutants compared to control mice (Figures 4.1 C, D). Paired pulse facilitation (PPF) analyses, indicative of the probability of synaptic release at presynaptic sites, showed comparable PPF ratios between *CaMKII-Wnt5a*$^{fl/fl}$ mice and their control littermates at both 3 and 6 months of age (Figures 4.2 A, B), suggesting that presynaptic neurotransmitter release is not altered with Wnt5a loss even at 6 months.

To assess the role of Wnt5a in NMDA receptor-dependent long-term potentiation (LTP), an electrophysiological correlate of strengthening of synaptic transmission, we used theta burst stimulation (TBS) to induce LTP at Schaffer collateral-CA1 synapses in 3 month-old mice. Recordings from *Wnt5a*$^{fl/fl}$ control slices revealed a robust induction of LTP and a sustained maintenance phase (Figure 4.3). In contrast, *CaMKII-Wnt5a*$^{fl/fl}$ slices showed a significant reduction in both induction and maintenance phases of LTP (Figures 4.3 A-C). Synaptic potentiation assessed by fEPSP slopes measured right after theta burst stimulation (4-14 min) was signficantly impaired in *CaMKII-Wnt5a*$^{fl/fl}$ mice, (274.3 ± 18.2 % in control slices *vs*. 201 ± 7.9 % in *CaMKII-Wnt5a*$^{fl/fl}$ slices, n=11 control and 13 mutant slices, 5 mice per genotype, p=0.0002, *t*-test), demonstrating an early requirement for Wnt5a in LTP induction. This effect of Wnt5a deletion was sustained as the responses remained attenuated 1 hr after theta burst stimulation in *CaMKII-Wnt5a*$^{fl/fl}$ slices (190.8 ± 13.3% and 151.9 ± 5.4% in control and mutant slices respectively, p=0.01, *t*-test). To address if Wnt5a contributes to NMDA receptor-dependent long-term depression (LTD), an electrophysiological correlate of weakening of synaptic transmission, we used a standard low frequency stimulation (LFS) paradigm to induce LTD in the CA1 hippocampus. In contrast to the LTP defect, we found no differences in LTD between mutant and control mice at 3 months of age (68.7 ± 2.9% and 68.2 ± 3% for control and *CaMKII-Wnt5a*$^{fl/fl}$ slices respectively; n= 14 and 16 slices for control and mutant slices respectively, 5 mice per genotype) (Figures 4.4 A, B).

Together, these results indicate an essential role for Wnt5a in the potentiation of synaptic efficacy. Notably, the impairment in LTP is detected at 3 months in *CaMKII-Wnt5a*$^{fl/fl}$ mice when neuronal structure and basal synaptic transmission are still intact.

**_CaMKII-Wnt5a$^{fl/fl}$_ mice exhibit impairments in recognition function and hippocampal dependent learning and memory**

Synaptic plasticity is widely considered to be a cellular mechanism that underlies learning and memory (Chen and Tonegawa, 1997; Whitlock et al., 2006). In addition, structural maintenance of synaptic connectivity has been postulated to be critical for life-long memories (Yang et al., 2009). Given the decreased CA1 LTP in 3 month old *CaMKII-Wnt5a$^{fl/fl}$* mice as well as structural abnormalities that are manifested in older *CaMKII-Wnt5a$^{fl/fl}$* mice at 6 months, we subjected Wnt5a mutant mice to behavioral paradigms to evaluate cognitive functions. To test recognition memory, we performed the novel object recognition test which evaluates the preference of mice for exploring a new over a familiar object (Bevins and Besheer, 2006). Adult *Wnt5a$^{fl/fl}$* and *CaMKII-Wnt5a$^{fl/fl}$* mice were allowed to freely explore a pair of identical objects and 24 hr later, were presented with the familiar object and a new object. Control mice showed a significant preference for the novel object over the familiar object, with the 3- and 6-month old mice spending 70.8 ± 5.5% and 68.1 ± 8.1% of time respectively exploring the new object. However, the *CaMKII-Wnt5a$^{fl/fl}$* mice showed no preference for the novel object over the familiar object at both 3 and 6 months of age, spending 51.4 ± 6.2% and 44.1 ± 7.4% of time, respectively, with the new object (Figures 4.5 A, B).

To test hippocampus-dependent spatial learning in these mice, we employed the Morris Water Maze (MWM) test, a classical behavioral task in which an animal's ability to remember spatial cues are used to locate a hidden platform in a tank of water (Vorhees and Williams, 2006) (Figure 4.7 A). First, we established that *CaMKII-Wnt5a$^{fl/fl}$* mice at both 3 and 6 months of age had visual acuity and swimming speeds comparable to litter-

mate controls (Figures 4.6 A, B). To evaluate their basic spatial learning ability, we first recorded the latency for each individual mouse to find the hidden platform for 12 consecutive days using 4 trials per day (see schematic in Figure 4.7 B). On day 13, to evaluate reference memory at the end of the learning, we conducted a probe trial in which mice were exposed to the same water maze but the platform was removed to determine if the mice exhibited a spatial bias toward the original target quadrant. Next, from days 14 to 25, we performed a reversal training by relocating the hidden platform to a new location (the quadrant opposite to the initial target quadrant), followed by probe trials from days 26 to 32 to assess memory retention (Figures 4.7 A, B).

During the naïve MWM training, 3 month old *CaMKII-Wnt5a*$^{fl/fl}$ mice took significantly longer time to locate the platform during the initial training period compared to age-matched controls, but eventually achieved similar latencies as control mice on the 6th day of training (Figure 4.7 C). 6 month old *CaMKII-Wnt5a*$^{fl/fl}$ mice also required significantly more time to find the platform compared to control mice, but did not reach the latency of control animals even when tested on the 12$^{th}$ day of the acquisition phase (Figure 4.7 D). During the probe trial on day 13 when the platform was removed, both 3- and 6-month old mutant mice spent significantly less time in the target quadrant (Figures 4.8 A, B), and made fewer crossings over the prior location of the submerged platform compared to control mice (Figures 4.8 C, D). During reversal learning from days 14 to 25 when the platform was relocated to the opposite quadrant, 3 month old mutant mice required more days of training to aquire a direct path to the new platform location compared to control mice, although they did achieve similar latencies as controls after 3 days of training (Figure 4.9 A). In contrast, 6 month old mutant mice required

significantly longer time to find the platform throughout the trials and did not catch up to their control litter-mates even by day 25 (Figure 4.9 B). In the following probe trial conducted from days 26 to 32 when the platform was removed, 3 month old mutant mice spent significantly less time in the target quadrant compared to controls, suggesting weaker spatial memory, although both mutant and control mice exhibited a distinct preference for the target quadrant compared to randomly spending 25% of time per quadrant throughout the 7 day test period (Figure 4.10 A). 6 month mutant mice also spent significantly less time in the target quadrant compared to controls, and markedly exhibited a decay in memory retrieval (Figure 4.10 B). While control animals exhibited a significant preference for the target quadrant even up to one week, this preference was lost in the mutants by the 5$^{th}$ day of the probe trial (Figure 4.10 B).

Together, these findings provide evidence for an essential role for Wnt5a in the acquisition of spatial learning and memory storage in adult animals. Notably, the cognitive dysfunction in 3 month old *CaMKII-Wnt5a*$^{fl/fl}$ mice were consistent with the LTP defects observed at this age. The more pronounced behavioral defects in older 6 month old *CaMKII-Wnt5a*$^{fl/fl}$ mice, which coincide with the manifestation of dendritic abnormalities, suggest a progressive decline in cognitive functions with age.

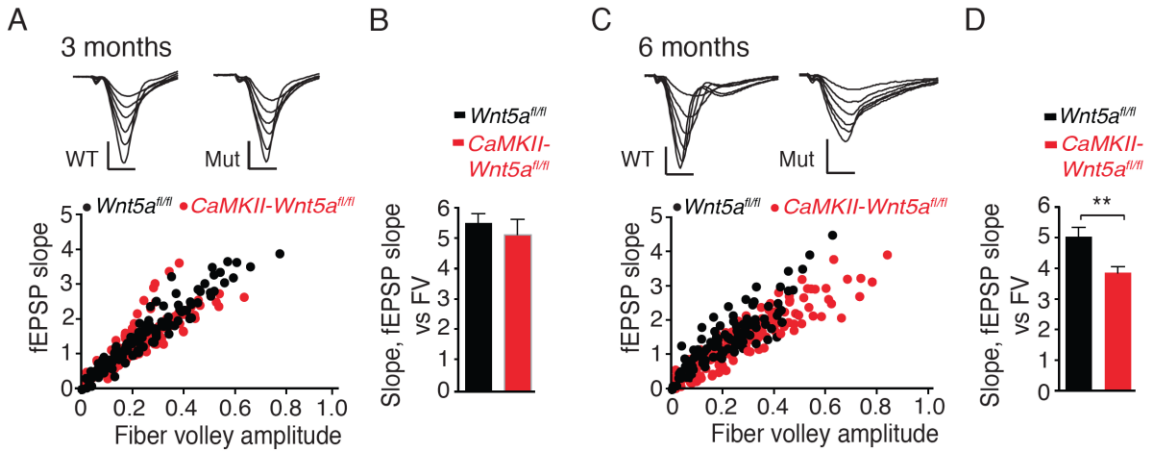



**Figure 4.1: Basal synaptic transmission is normal in 3 month *CaMKII-Wnt5a$^{fl/fl}$* mice but decreased in 6 month adult *CaMKII-Wnt5a$^{fl/fl}$* mice.**

**(A)** Basal synaptic transmission is unaffected in *CaMKII-Wnt5a$^{fl/fl}$* mice at 3 months. There are no differences in the input-output relationship between control and mutant mice at 3 months of age. (**B**) The slope of the input-output curve is not altered in 3 month old *CaMKII-Wnt5a$^{fl/fl}$* mice. n=18 and 17 slices from 5 animals per genotype. Scale bars: 1 mV (vertical), 2.5 ms (horizontal) for both sample traces. (**C**) Basal synaptic transmission is impaired in 6 month old *CaMKII-Wnt5a$^{fl/fl}$* mice. **(D)** The slope of the input-output curve shows a decrease in 6 month old *CaMKII-Wnt5a$^{fl/fl}$* mice compared to control mice. Results are mean ± SEM from 5 *Wnt5a$^{fl/fl}$* (n=21 slices) and 4 *CaMKII-Wnt5a$^{fl/fl}$* mice (n=23 slices), **p<0.01, unpaired, two-tailed *t* test. Scale bars: 1 mV (vertical), 2.5 ms (horizontal) for both sample traces.

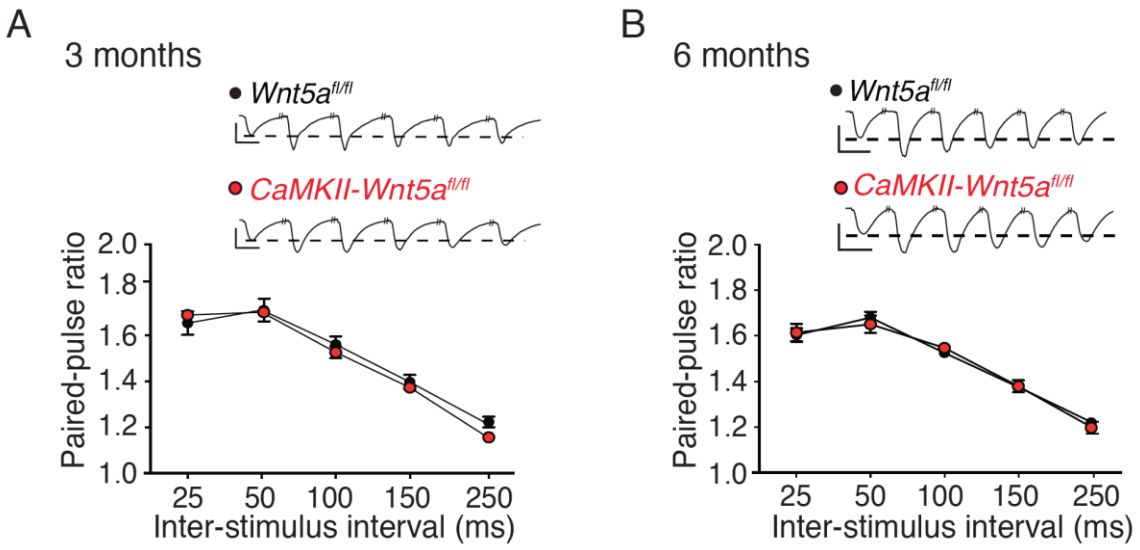



**Figure 4.2. Pre-synaptic properties are unaltered in *CaMKII-Wnt5a$^{fl/fl}$* mice.**

**(A, B)** Pre-synaptic vesicle release probablity are normal in 3 and 6 month old *CaMKII-Wnt5a$^{fl/fl}$* mice as measured by paired-pulse ratios with different inter-stimulus intervals (ms). Results are means ± SEM from 4 animals per genotype for 3 month old animals (n=17 slices for *Wnt5a$^{fl/fl}$* and n=15 slices from *CaMKII-Wnt5a$^{fl/fl}$* mice). For 6 month old animals, 4 *Wnt5a$^{fl/fl}$* mice (n=24 slices) and 3 *CaMKII-Wnt5a$^{fl/fl}$* mice (n=22 slices) were used. Scale bars for sample traces in insets; 1 mV (vertical), 10 ms (horizontal). Statistical analyses were done using a two-way ANOVA.

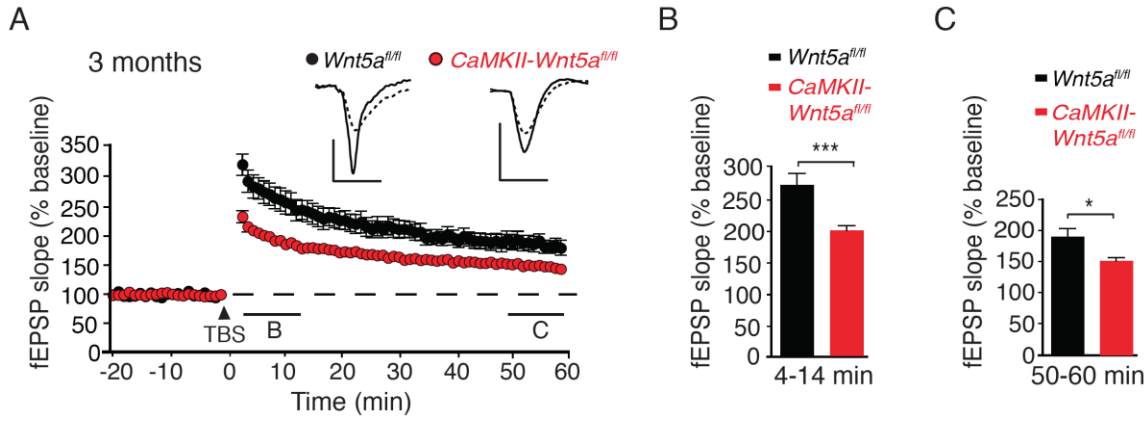



**Figure 4.3: 3 month old *CaMKII-Wnt5a$^{fl/fl}$* mice have Long-term potentiation (LTP) deficit.**

**(A)** Impaired LTP at the Schaffer collateral-CA1 synapses in 3 month old *CaMKII-Wnt5a$^{fl/fl}$* mice. n=11 slices from 5 *Wnt5a$^{fl/fl}$* mice and n=13 slices from 6 *CaMKII-Wnt5a$^{fl/fl}$* mice. Sample traces represent fEPSPs right before (dashed line) and 1 hour after (solid line) theta burst stimulation. Scale bars: 1 mV (vertical), 10 ms (horizontal) for both sample traces. **(B)** LTP induction is reduced in 3 month old *CaMKII-Wnt5a$^{fl/fl}$* mice. Results are mean ± SEM from 5 *Wnt5a$^{fl/fl}$* mice (n=11 slices) and 6 *CaMKII-Wnt5a$^{fl/fl}$* mice (n=13 slices). ***$p<0.001$, unpaired, two-tailed *t* test. **(C)** LTP maintenance is attenuated in 3 month old *CaMKII-Wnt5a$^{fl/fl}$* mice. Results are mean ± SEM from 5 *Wnt5a$^{fl/fl}$* mice (n=11 slices) and 6 *CaMKII-Wnt5a$^{fl/fl}$* mice (n=13 slices) *$p<0.05$, unpaired, two-tailed *t* test**.**

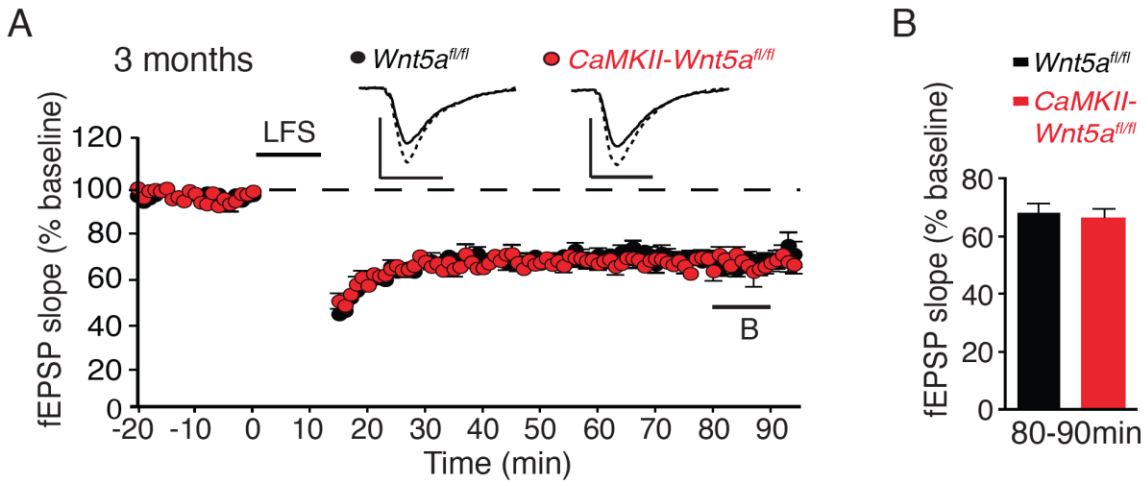



**Figure 4.4: 3 month old *CaMKII-Wnt5a$^{fl/fl}$* mice show comparable Long-term depression (LTD).**

**(A)** Low frequency stimulation-induced LTD at Schaffer collateral-CA1 synapses is unaltered in 3 month *CaMKII-Wnt5a$^{fl/fl}$* mice. Sample traces represent fEPSPs right after (dashed line) and 1hr after (solid line) stimulation. Scale bars: 1 mV (vertical), 10 ms (horizontal) for both sample traces. **(B)** Mean fEPSP slopes are comparable between the genotypes. Results are mean ± SEM from 5 *Wnt5a$^{fl/fl}$* mice (n=14 slices) and 5 *CaMKII-Wnt5a$^{fl/fl}$* mice (n=16 slices).

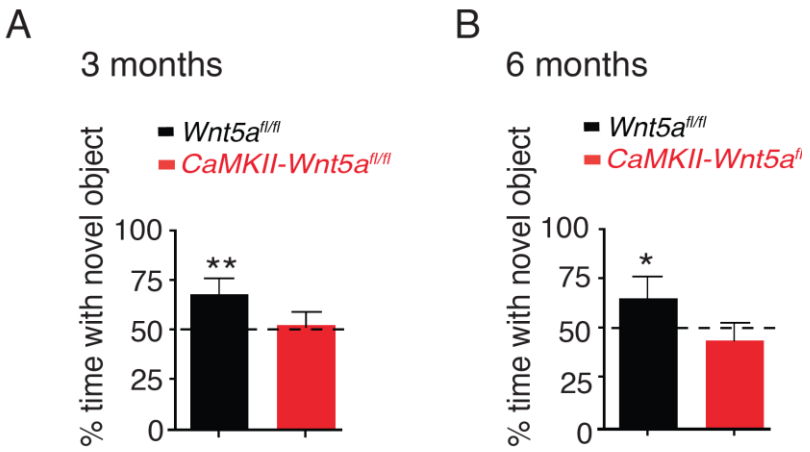



**Figure 4.5: Both 3 month and 6 month *CaMKII-Wnt5a$^{fl/fl}$* mice mice show deficits in novel object recognition task.**

**(A, B)** Control *Wnt5a$^{fl/fl}$* showed a significant preference for a new object in the novel object recognition task, while *CaMKII-Wnt5a$^{fl/fl}$* mice spent similar amounts of time exploring the familiar and new objects. Deficits in recognition memory were evident in both 3- and 6-month old mutant animals. Dashed line indicates equal amount of time spent exploring new and familiar objects. Results are mean ± SEM from n=21 *Wnt5a$^{fl/fl}$* mice and n=19 *CaMKII-Wnt5a$^{fl/fl}$* mice at 3 months, and n=16 *Wnt5a$^{fl/fl}$* and n=23 *CaMKII-Wnt5a$^{fl/fl}$* at 6 months. *$p<0.05$, **$p<0.01$ significantly different from 50% time spent with novel object, unpaired, two-tailed *t* test.

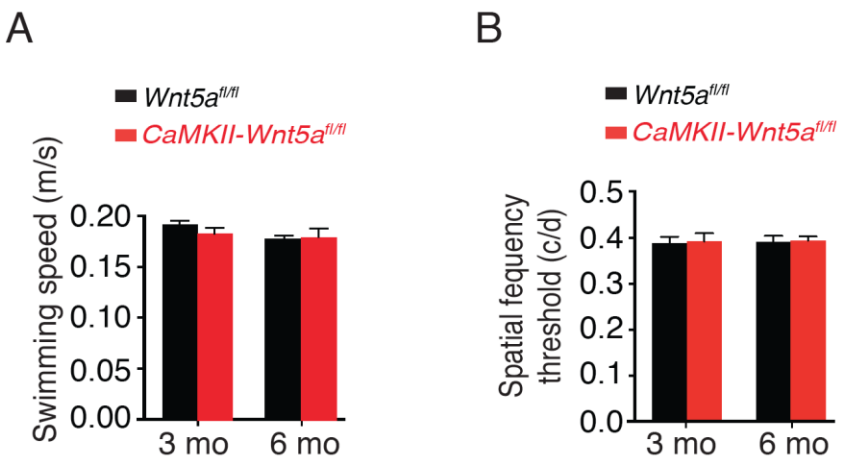



**Figure 4.6: Both 3 month and 6 month *CaMKII-Wnt5a$^{fl/fl}$* mice show normal swimming speed and vision acuity.**

**(A, B)** Swimming speeds and visual acuities are similar between *CaMKII-Wnt5a$^{fl/fl}$* mice and control litter-mates at both 3 and 6 months of age. Results are means ± SEM from 20 *Wnt5a$^{fl/fl}$*, 19 *CaMKII-Wnt5a$^{fl/fl}$* mice at 3 months of age and 17 *Wnt5a$^{fl/fl}$*, 20 *CaMKII-Wnt5a$^{fl/fl}$* mice at 6 months.

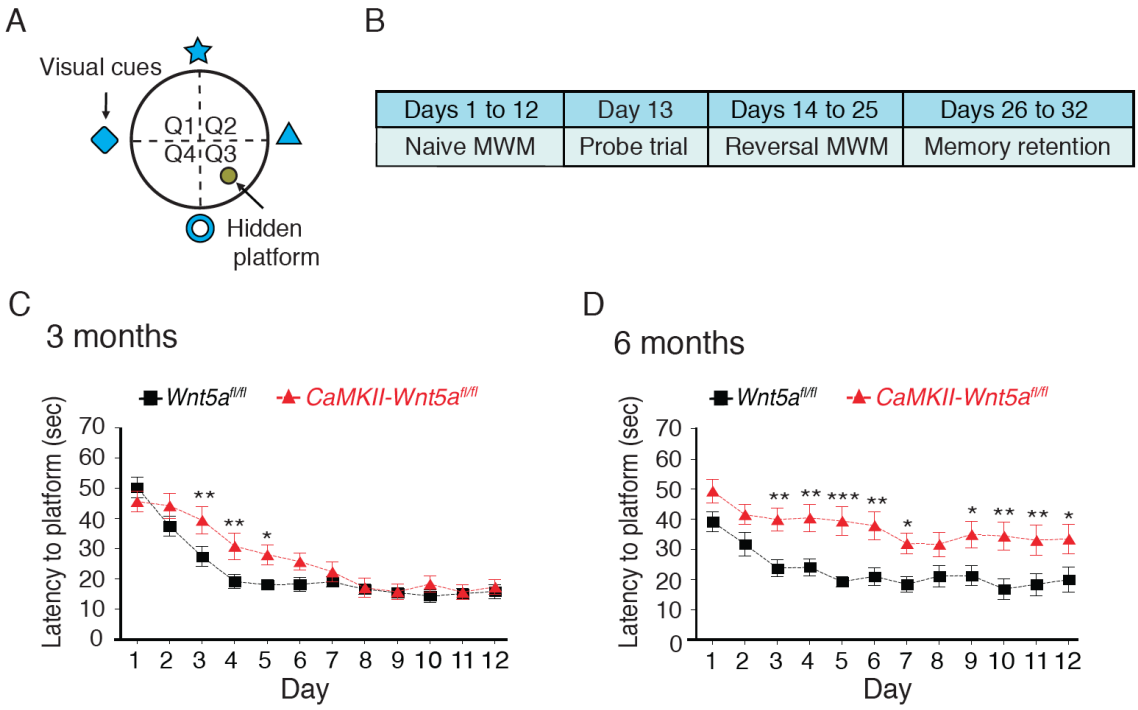



**Figure 4.7: *CaMKII-Wnt5a$^{fl/fl}$* mice exhibit a progressive cognitive functions decline in hippocampal dependent Morris water maze (MWM) learning and memory assay.** **(A)** Schematic of the Morris water maze (MWM). **(B)** Timeline for the MWM tasks. **(C)** Impaired learning in 3 month old *CaMKII-Wnt5a$^{fl/fl}$* mice. Results are mean ± SEM from n=20 *Wnt5a$^{fl/fl}$* and n=19 *CaMKII-Wnt5a$^{fl/fl}$* mice at 3 months, *p<0.05; **p<0.01, two-way ANOVA. **(D)** 6 month *CaMKII-Wnt5a$^{fl/fl}$* mice exhibit severe deficits in learning and fail to acquire the latency of control animals even on day 12. Results are mean ± SEM from n=18 *Wnt5a$^{fl/fl}$* and n=20 *CaMKII-Wnt5a$^{fl/fl}$* mice at 6 months of age. *p<0.05, **p<0.01, ***p<0.001, two-way ANOVA.

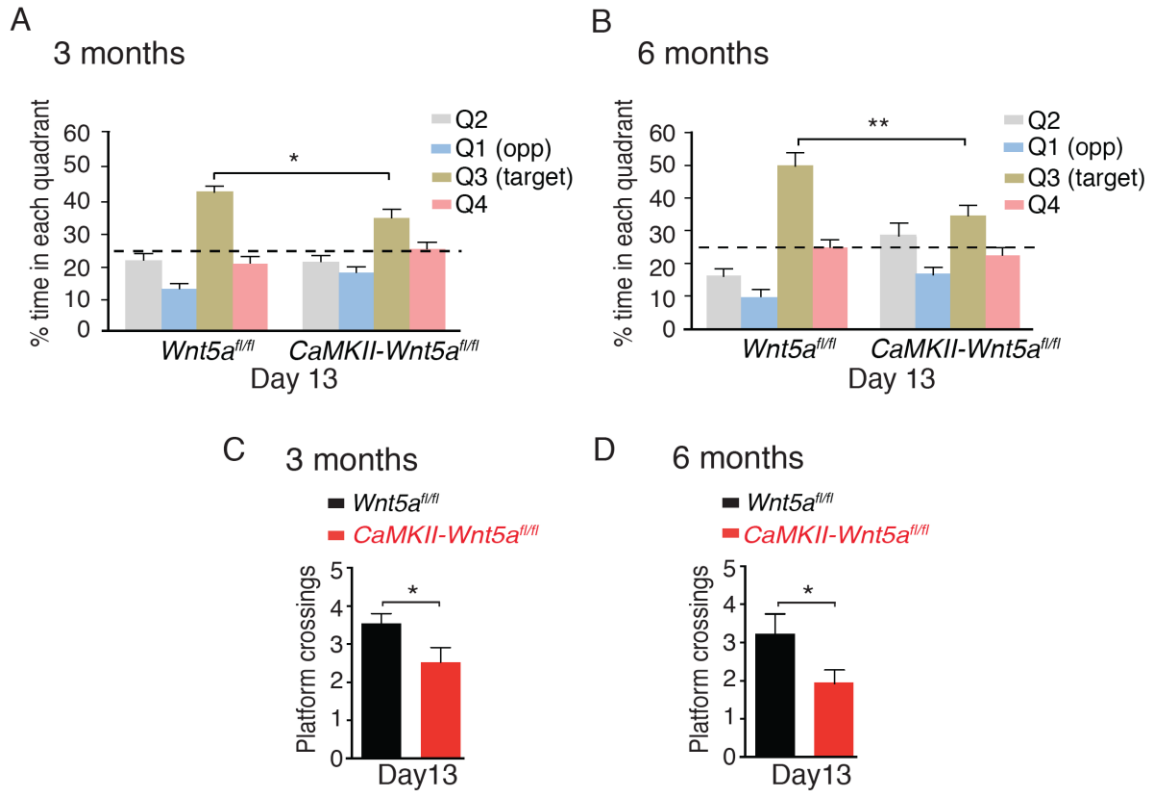



**Figure 4.8: Both 3 month and 6 month *CaMKII-Wnt5a$^{fl/fl}$* mice show memory deficit in MWM probe trial test.**

**(A, B)** During probe trials**,** 3 month and 6 month *CaMKII-Wnt5a$^{fl/fl}$* mice showed less preference for the target quadrant compared to control litter-mates. Results are mean ± SEM, *p<0.05, **p<0.01 unpaired two-tailed *t* test. **(C, D)** 3 and 6 month old *CaMKII-Wnt5a$^{fl/fl}$* mice exhibit fewer crossings over the original platform location during the probe trial on day13, compared to control litter-mates. Results are means ± SEM from n=20 *Wnt5a$^{fl/fl}$* and n=19 *CaMKII-Wnt5a$^{fl/fl}$* mice at 3 months, and from n=18 *Wnt5a$^{fl/fl}$* and n=20 *CaMKII-Wnt5a$^{fl/fl}$* mice at 6 months. *p<0.05, unpaired two-tailed *t* test.

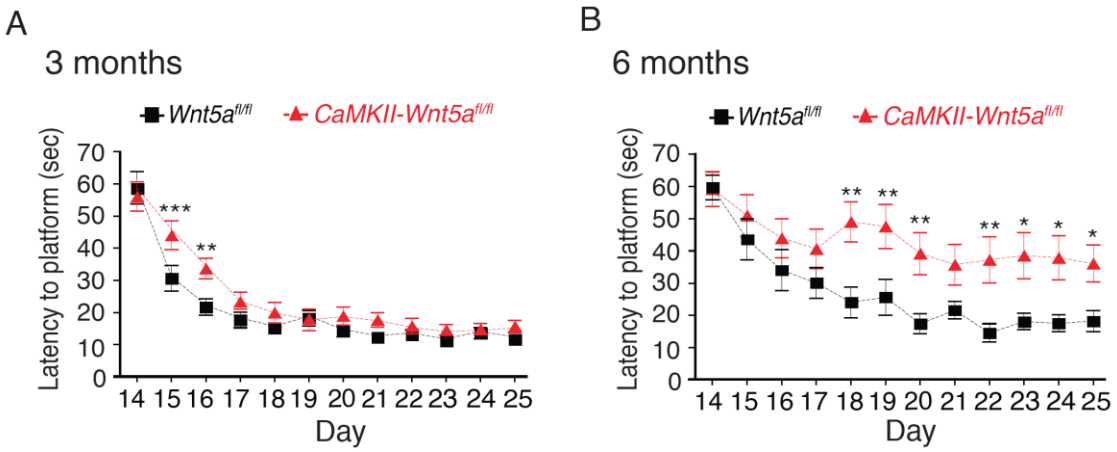



**Figure 4.9: 6 month *CaMKII-Wnt5a$^{fl/fl}$* mice show severe deficits in reversal MWM learning task.**

**(A, B)** Both 3 and 6 month-old *CaMKII-Wnt5a$^{fl/fl}$* mice showed deficits in reversal learning compared to control mice, and the deficits were more pronounced in 6 month old mutant animals. *p<0.05, **p<0.01, ***p<0.001, two-way ANOVA.

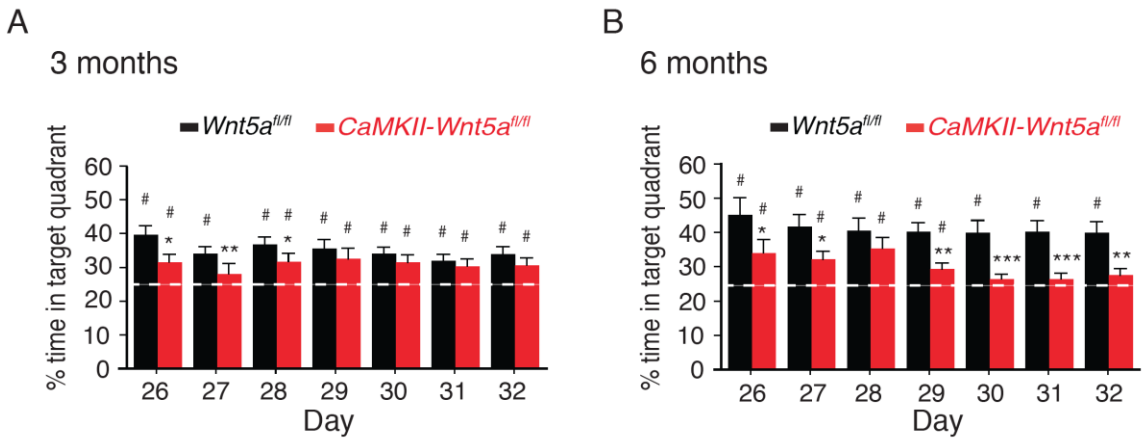



**Figure 4.10: 6 month *CaMKII-Wnt5a$^{fl/fl}$* mice have severe impairment in memory retention.**

**(A)** During the memory retention test, 3 month old *CaMKII-Wnt5a$^{fl/fl}$* mice spent less time in the target quadrant compared to control littermates. White dashed line indicates 25% of time that mice spent in the target quadrant. Results are mean ± SEM, # $p<0.05$ significantly different from 25%**,** *$p<0.05$, **$p<0.01$ significantly different from control litter-mates, unpaired two-tailed *t* test**.** (**B**) 6 month old *CaMKII-Wnt5a$^{fl/fl}$* mice also spent significantly less time in the target quadrant compared to controls, but exhibited a marked decay in memory retrieval by the 5$^{th}$ day of the probe trial. White dashed line indicates 25% time mice spent in the target quadrant. Results are means ± SEM, # $p<0.05$ significantly different from 25%**,** *$p<0.05$, **$p<0.01$, ***$p<0.001$ significantly different from control litter-mates, unpaired two-tailed *t* test.

## Discussion

Wnts are secreted glycoproteins that have critical and evolutionarily conserved functions during embryonic development (Logan and Nusse, 2004). In the nervous system, Wnt5a play a critical roles in neuronal morphogenesis during early development and synapse formation in cultured hippocampal pyramidal neurons (Bian et al., 2015; Blakely et al., 2011; Bodmer et al., 2009; Cuitino et al.; Davis et al., 2008; Li et al., 2009; Rodriguez-Gil and Greer, 2008; Shafer et al., 2011; Varela-Nallar et al., 2010a; Zhang et al., 2007). Interestlying, we identified that Wnt5a protein expression pattern starts at postnatal stage and last to adulthood in the hippocampus. However, less is known about the functions of Wnt5a in the adult animals, due to global developmental defects and the prenatal lethality in conventional Wnt5a knockout mice (Yamaguchi et al., 1999).

With generating neuron-specific deletion of *Wnt5a* in *CaMKII-Wnt5a*$^{fl/fl}$ mice, we reveal an essential role for Wnt5a in maintaining dendrite morphology in the adult hippocampus (Figures 2.12 B,C and D; Figures 2.17 Band D). Loss of Wnt5a in hippocampal CA1 pryamidal neurons does not compromise dendrite development, but results in striking adult-onset defects in dendritic complexity, lengths and spine densities that manifest in 6 months-old mutant mice. Electrophysiological analyses of 3 month-old *CaMKII-Wnt5a*$^{fl/fl}$ mice which have normal dendritic complexity and spine density as wild type littermates revealed impaired synaptic plasticity in LTP. By applying paired-pulse ratio assay to analyze the spynaptic vesicles release probability from pre-synaptic terminal shown similar faciliation response. This evidence indicate Wnt5a regulate synaptic plasticity mainly on the post-synaptic terminal but not the pre-synaptic terminal.

We further tested synaptic strength of CA1 pyramidal neurons from acute brain slices of both 3 month and 6 month-old *CaMKII-Wnt5a*$^{fl/fl}$ mice by extracellular recorded population excitatory postsynaptic potentials (EPSPs). By measuring the input/output (I/O) curve, we found a significant decrease in synaptic efficacy in 6 month-old, *CaMKII-Wnt5a*$^{fl/fl}$ hippocampal slices. These results indicate that Wnt5a has function in regulating either synapse number or glutamate receptor number in the post-synaptic terminal in the hippocampus.

Synaptic plasticity is critical for information processing in the brain and has been shown to underlie many complex behaviors including learning and memory. We found that synaptic plasticity compromises in 3 month- old *CaMKII-Wnt5a*$^{fl/fl}$ mice in novel object recognition and slight deficits in Morris Water Maze learning and memory test. To note that 3 month- old *CaMKII-Wnt5a*$^{fl/fl}$ mice have normal dendritic complexity and spine density as wild type littermates. Remarkably, due to progressively decline with the manifestation of dendritic structure abnormalities, 6 month-old *CaMKII-Wnt5a*$^{fl/fl}$ mice showed severe defects in Morris Water Maze learning and memory test. Together, These findings provide the first direct genetic evidence for a Wnt protein in adult animal behavior in the acquisition of spatial learning and memory storage.

## Methods

### Animals

All procedures relating to animal care and treatment conformed to institutional and NIH guidelines. Animals were housed in a standard 12:12 light-dark cycle. The generation of *Wnt5a*$^{fl/fl}$ mice has been previously described (Ryu et al., 2013). *Wnt5a*$^{fl/fl}$ mice were backcrossed to *C57BL/6* background for at least 7 generations and maintained on a *C57BL/6* background. *Nestin-Cre* and *TOPGAL* mice were obtained from Jackson Laboratory and *CaMKII-Cre* mice (T29-1 line) were a generous gift from Dr. Nicholas Gaiano. Sprague Dawley rats were purchased from Charles River. Hippocampal neuron cultures were established from embryonic day 18 (E18) rat pups as previously described (Araki et al., 2015).

### Slice preparations and electrophysiology

3 or 6 month-old male mice were anesthetized with the inhalation anesthetic isoflurane prior to decapitation. Brains were rapidly dissected out and placed in ice-cold, oxygenated (95% $O_2$ and 5% $CO_2$) low-$Ca^{2+}$/high-$Mg^{2+}$ dissection buffer containing 2.6 mM KCl, 1.25 mM $NaH_2PO_4$, 26 mM $NaHCO_3$, 211 mM sucrose, 11 mM glucose, 0.5 mM $CaCl_2$ and 7 mM $MgCl_2$. 350 □m transverse hippocampal slices were prepared using a vibratome (Leica; VT1000s) in dissection buffer followed by recovery in a static submersion chamber filled with oxygenated artificial cerebrospinal fluid (ACSF) containing 125 mM NaCl, 5 mM KCl, 1.25 mM $NaH_2PO_4$, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM $NaHCO_3$ and 11 mM glucose at 30 °C for at least 1 hour before LTP or 2 hr before LTD recording.

Slices were transferred to a recording chamber where they were perfused continuously with oxygenated ACSF at a flow rate of ~3 ml/min at 30 ◦C. Hippocampal CA1 field excitatory postsynaptic potential (fEPSP) was evoked at 0.033 Hz with a 125 μm platinum/ iridium concentric bipolar electrode (FHC, Bowdoinham, ME) placed in the middle of stratum radiatum of CA1. Synaptic responses were recorded with ACSF-filled microelectrodes (1–2MΩ), positioned ~200 μm away (orthodromic) from the stimulating electrode, and were quantified as the initial slope of fEPSP. Input-output relationships were obtained for each slice with various stimulus intensity and responses were set to ~45% max for LTP experiments and ~55% max for LTD experiments. Input-output curves were plotted as the fEPSP slope against the presynaptic fiber volley amplitude across all stimulation intensities and the slope for the linear fit of the fiber volley–fEPSP slope relationship were obtained from each slice. Paired-pulse facilitation (PPF) was recorded with inter-stimulus interval of 25−250 ms. PPF data were presented as a ratio of the second response slope relative to the first. LTP was induced by theta burst stimulation (TBS) consists of 4 trains of 10 bursts at 5 Hz, with each burst consisting of four stimuli given at 100 Hz and 10 second inter-train interval. LTD was induced by long frequency stimulation (LFS) consists of 900 single pulses at 1 Hz.

All hippocampal slice electrophysiological recordings were performed by an experimenter blind to the animal genotype. Statistical significance was determined with a two-way ANOVA for PPF and a two-tailed, unpaired *t* test for input/output relationship, LTP and LTD. Representative traces are averages of 4 (PPF and input/output relationship) or 10 (LTP and LTD) traces, and stimulus artifacts have been removed for clarity.

## Behavioral tests

### Morris water maze

Mice were trained to find a submerged platform in a water maze in a 12-day training protocol as previously described (LeGates et al., 2012). Mice were tested in four stages: naïve acquisition, probe trial, reversal learning, probe trials for memory retention (see Figure 4.7 B). During the naïve acquisition phase, mice were trained (four trials per day for 12 days) to find a hidden platform using four visual distal cues equally surrounding the pool. Each mouse was randomly placed in a different area of the pool at the start of each trial with the platform maintained in the same quadrant (target quadrant). The platform was then removed on day13 during the probe trial. Swimming in each quadrant and specifically the preference for the target quadrant was measured to evaluate spatial memory using a computerized video tracking system (Any-maze) with a camera mounted in the center above the pool. Reversal training began on day14 when the platform was moved to the quadrant opposite to the original target quadrant. Mice were trained as described for the acquisition phase.

Latency to locate the platform during the acquisition and reversal phases were analyzed by two-way ANOVA followed by a Fisher-LSD post-hoc test to examine changes in latency throughout the course of the experiment as well as the effect of the genotype. Probe trials were analysed by calculating the percentage time spent in the target quadrant and performing a two-tailed *t*-test.

### Novel object recognition

Recognition memory was assessed using the novel object recogniton test. Mice were first removed from their home cages, acclimated to the empty testing arena for 10 min, and subsequently returned to their home cages. The day after acclimation, mice were returned to this arena with two identical objects that they could freely explore for 10 min, after which they were returned to their home cages for 24 hr. At the end of the 24 hr period, mice were placed back into the arena with one of the objects changed to a novel object, and were allowed to explore both the familiar and novel objects for 5 min. Behavior was monitored from above by a video camera connected to a computerized video tracking system (Any-maze), and the percentage of time spent with each object was calculated. The objects and arena were thoroughly cleaned between each trial to remove odor cues. Object recognition was analysed by calculating the percentage time spent with the novel object and performing a one sample *t*-test to determine whether this was significantly above 50%.

**Visual acuity**

Visual acuity was assessed by measuring the image-tracking reflex in a virtual cylinder "OptoMotry" (Cerebral Mechanics, Lethbridge, Canada) as previously described (Guler et al., 2008). A sine wave grating was projected on the screen rotating in a virtual cylinder. The animal was assessed for a tracking response upon stimulation for approximately 5 seconds. All acuity thresholds were determined using the staircase method with 100% contrast.

**Statistical analyses**

All Student's *t* tests were performed using two-tailed, unpaired, and a confidence interval of 95%. One-way or two-way ANOVA analyses were performed when more than two groups were compared. Statistical analyses were based on at least 3 independent experiments, and described in the figure legends. All error bars represent the standard error of the mean (s.e.m).

# Chapter 5: Wnt5a signaling in the adult hippocampus

## Introduction

The Wnt protein family, which includes 19 members in mammals, all function as secreted glycoproteins and are reported to have morphogenetic activity. With the ability to initiate many signaling cascades, disruption of Wnt signaling has resulted in various diseases, including cancers, melanoma (Polakis, 2012), and neurodegenerative diseases (Inestrosa and Arenas, 2010). Wnt proteins have been classified as two major groups, canonical and non-canonical pathways, from their properties to initiate downstream signaling (Gordon and Nusse, 2006). Based on recent study, Wnt1, Wnt3a, Wnt7a/b, and Wnt8 are identified as common activators for the canonical wnt pathway, whereas Wnt4, Wnt5a, and Wnt11 are belonged to the non-canonical Wnt pathway (Gordon and Nusse, 2006; Kikuchi et al., 2011; MacDonald et al., 2009).

### The Canonical pathway of β-Catenin dependent pathway

Wnt binds to Fz and LRP5/6 receptor complex that activates the cytoplasmic protein disheveled (Dvl), which serves as a platform for the recruitment of the β-catenin destruction complex (Bilic et al., 2007). This β-catenin destruction complex includes the scaffold protein Axin, Glycogen synthesis kinase-3β (GSK- 3β), Casein kinase-1 α (CK-1α), and the tumor suppressor adenomatous polyposis coli (APC) (Cliffe et al., 2003; Gao and Chen, 2010; Schwarz-Romond et al., 2007). Once Wnt binds to Fz and LRP5/6 receptor complex, CK-1α phosphorylates LRP5/6 which inhibits the β- catenin destruction complex. Thus, Wnts prevent β-catenin from degradation and allows it to translocate to the nucleus. In the nucleus, β-catenin interacts with other TCF/LEF transcription factors to activate the transcription of Wnt target genes (Clevers and Nusse,

2012; Logan and Nusse, 2004). There are several genes invloved in cell proliferation and signaling activation which are transcribed by β-catenin/TCF/LEF complex, including c-Myc, cyclin D1, Axin2, and $Ca^{2+}$-calmodulin-dependent protein kinase type IV (Arrazola et al., 2009; Hodar et al., 2010; Nusse and Varmus, 2012).

**The Non-canonical pathway or β-Catenin-independent pathway**

Two signaling cascades activated by Wnt ligands are grouped as non-canonical pathway which involves downstream effectors that are independent of β-catenin signaling: the planar cell polarity (PCP) pathway and the calcium pathway. The Wnt/PCP pathway is first identified for its functions in regulating tissue polarity and cell migration during development in *Drosophila* (Adler, 2002; Veeman et al., 2003). In this pathway, the binding of Wnt to the Fz receptors activates Rho and Rac, which in turn stimulates Rho-associated protein kinase (ROCK) and JNK, respectively. The Rho and Rac are small GTPases important for regulating actin polymerization, cytoskeletal organization, spine morphologies and cell motility. In addition, another Wnt/PCP pathway downstream effector, c-JunN-terminal kinase (JNK) are important in controling cell polarity as well as gene transcription.

The other non-canonical pathway- Wnt/Calcium pathway acts mainly through a G-protein-dependent signaling cascade (Kohn and Moon, 2005; Slusarski et al., 1997a; Slusarski et al., 1997b). Once Wnt binds to Fz, the seven transmembrance receptor will stimulate heterotrimeric G-proteins, which activates phospholipase-C (PLC). As a result, PLC is able to generate diacylglycerol (DAG) and inositol-1,4,5-thriphosphate (IP3). IP3 interacts with the Inositol triphosphate receptor which acts as calcium channels present

on the membrane of endoplasmic reticulum (ER) resulting in release of calcium (Kohn and Moon, 2005). Calcium further activate calcium calmodulin dependent protein kinase II (CaMKII). DAG produced by hydrolysis of phosphatidyl inositol 4,5-bisphosphate and together with elevated calcium activates protein kinase C (PKC) (Kuhl et al., 2000a; Kuhl et al., 2000b; Sheldahl et al., 1999). Both CaMKII and PKC in turn activate epigenetic modification enzyme suh as histone deacetylase (HDAC) and various nuclear transcription factors such as NFκB and CREB (Kim et al., 2012; La Porta and Comolli, 1998; Ma et al., 2011). Depending on the calcium concentration released from ER, the non-cannonical pathway can also activate calcinurin, a protein phosphatase, that can dephosphorylate downstream efectors and in turn activate Nuclear Factor Associated with T cells (NFAT) (Spinsanti et al., 2008). Interestingly, different Wnt ligands compete for the Fz receptor at the cell surface and may cause a inhibition of the reciprocal pathway providing another regulaiton in determining the specificity of signaling (Grumolato et al., 2010).

## Result

Wnts are known to exert their effects by signaling through three effector pathways; the canonical β-catenin-dependent pathway, a $Ca^{2+}$-dependent pathway and the planar cell polarity (PCP) pathway (Ciani and Salinas, 2005). To define Wnt5a-mediated signaling events in the hippocampus, we assessed biochemical changes in young adult *Nestin-Wnt5a*$^{fl/fl}$ mice at 1.5 months before the appearance of structural anomalies.

We first assessed whether Wnt5a deletion affects canonical Wnt signaling by crossing *Nestin-Wnt5a*$^{fl/fl}$ mice with *TOPGAL* transgenic mice, where *LacZ* expression reports β-catenin-dependent transcriptional activity (DasGupta and Fuchs, 1999). We observed comparable X-gal staining in the hippocampus in 1.5 month old *TOPGAL;Nestin-Wnt5a*$^{fl/fl}$ and control *TOPGAL;Wnt5a*$^{fl/fl}$ mice (Figure 5.1 A). Biochemical assays revealed similar levels of accumulated β-catenin in nuclear fractions from *Nestin-Wnt5a*$^{fl/fl}$ and control hippocampus (Figures 5.1 B and C). We also performed qPCR analysis to assess the expression of *Axin2*, a well-established transcriptional target of canonical β-catenin signaling (Yan et al., 2001). We found comparable *Axin2* transcript levels between Wnt5a mutant and control hippocampi (Figure 5.1 D). These results indicate that canonical Wnt signaling in the mature hippocampus is unaffected by Wnt5a depletion.

We next assessed the Wnt-calcium pathway where Wnt ligands promote an increase of cytoplasmic $Ca^{2+}$ (Kohn and Moon, 2005; Veeman et al., 2003). We performed calcium imaging in cultured rat hippocampal neurons acutely treated with Wnt5a. Hippocampal neurons transfected with GCaMP3, a genetically encoded calcium reporter, were treated with Wnt5a conditioned or control media diluted in artificial cerebrospinal

fluid (ACSF) buffer and delivered via a continuous perfusion. Strikingly, Wnt5a elicited a calcium response in 92% of the neurons, while only 43% of neurons responded to control media. Furthermore, the number of calcium transients was 5-fold higher in Wnt5a-treated neurons (Figures 5.2 A-C). The average frequency of the calcium transients was 12.04 ± 1.4 per 30 minutes in Wnt5a-treated neurons compared to 2.27 ± 0.5 in control neurons (Figure 5.2 C).

Wnt-calcium signaling activates $Ca^{2+}$-sensitive enzymes including calcium-calmodulin kinase II (CaMKII) and protein kinase C (PKC) (Kohn and Moon, 2005; Veeman et al., 2003), which are also known to be critical regulators of hippocampal connectivity and functions (Sweatt, 1999; Wayman et al., 2008). We thus performed immunoblotting analyses of hippocampal homogenates using a phospho-specific antibody that reports CaMKII activation (threonine 286 phosphorylation on CaMKII-α and threonine 287 phosphorylation on CaMKII-β) (Lisman et al., 2002). At 2 weeks of age when dendrite branch elaboration is on-going, there were no changes in the levels of phosphorylated CaMKII in *Nestin-Wnt5a*$^{fl/fl}$ mice (Figures 5.2 D and E), although Wnt5a depletion was near-complete by this time (Figures 3.3). These results suggest that CaMKII signaling in the developing hippocampus is not altered with Wnt5a depletion. However, at 1.5 months of age, we observed a pronounced attenuation of phosphorylated CaMKII-α (57% decrease) and CaMKII-β (40% decrease) in *Nestin-Wnt5a*$^{fl/fl}$ mice (Figures 5.2 F and G). A modest yet significant decrease (10%) in PKC phosphorylation was also observed in the Wnt5a mutant hippocampus at 1.5 months of age (Figures 5.3 A and B).

Calcium signaling plays a pivotal role in regulating dendritic structure and synaptic functions in the hippocampus (Lisman et al., 2002; Wong and Ghosh, 2002). In post-synaptic density fractions isolated from *Nestin-Wnt5a*$^{fl/fl}$ hippocampus, we observed a marked decrease in phosphorylation of the GluA1 subunit of AMPA-type glutamate receptors at a critical Serine 831 site, known to be phosphorylated by both CaMKII and PKC (Mammen et al., 1997; Roche et al., 1996), (Figures 5.4 A and B). Serine 831 phosphorylation has been functionally linked to channel conductance, synaptic plasticity and retention of spatial memory in mice (Derkach et al., 1999; Lee et al., 2003).

Calcium signaling within synapses can couple to transcriptional responses via shuttling of a $Ca^{2+}$/CaM/CaMKII-γ complex to the nucleus to promote phosphorylation of CaMKIV which then phosphorylates and activates the transcription factor CREB (Ma et al., 2014). We found that phosphorylation of CaMKIV was significantly reduced by 39% (Figures 5.5 A and B) while CREB phosphorylation was decreased by 59% in nuclear fractions prepared from 1.5 month old *Nestin-Wnt5a*$^{fl/fl}$ hippocampal tissues (Figures 5.5 C and D). However, at 2 weeks of age, there were no differences in CaMKIV or CREB phosphorylation levels between mutant and control mice (Figures 5.5 E-H). These results suggest that Wnt5a functions via a $Ca^{2+}$-mediated signaling cascade that impinges on CREB phosphorylation in mature hippocampal neurons.

We also examined the planar cell polarity pathway where non-canonical Wnts induce cytoskeletal dynamics by activating small GTPases, such as Rac1, and JNK signaling (Montcouquiol et al., 2006). Rac1 is a critical regulator of the actin cytoskeleton in dendrites and spines (Luo, 2002; Van Aelst and Cline, 2004). Indeed, we found that active Rac1-GTP was reduced by 51% in hippocampal homogenates from 1.5 month

*Nestin-Wnt5a*$^{fl/fl}$ mice, although total Rac1 protein levels were unaffected (Figures 5.6 A and B). There were no changes in Rac1 activity in the *Nestin-Wnt5a*$^{fl/fl}$ hippocampus at 2 weeks of age (Figures 5.6 C and D). Phospho-JNK levels were also significantly reduced in hippocampal lysates from *Nestin-Wnt5a*$^{fl/fl}$ mice at 1.5 months but not at 2 weeks of age (Figures 5.7 A-D). Rac1 activity can also be influenced by CaMKII activity via CaMKII-mediated phosphorylation of the Rac1-specific GEFs, Tiam1 and Kalirin-7 (Tolias et al., 2005; Xie et al., 2007).

In *CaMKII-Wnt5a*$^{fl/fl}$ mice, we also found that phosphorylation of CaMKII and GluA1 (Ser-831) (Figures 5.8 A-D) as well as Rac1 activity (Figures 5.9 A and B) and CREB phosphorylation (Figures 5.10 A and B) were significantly diminished in hippocampal homogenates at 3 months of age, similar to that observed in 1.5 month-old *Nestin-Wnt5a*$^{fl/fl}$ mice. Of note, at 3 months of age, there are no structural abnormalities in *CaMKII-Wnt5a*$^{fl/fl}$ mice ( Figures 3.11 A and C; Figures 3.12 A and B).

Together, these analyses suggest that loss of Wnt5a impairs calcium and cytoskeleton-associated biochemical cascades that are essential for synaptic structure and plasticity in the mature hippocampus.

**Wnt5a regulates CREB-dependent GluN1 synthesis**

Our finding that Wnt5a deletion altered nuclear responses, including decreased CREB phosphorylation, suggested a role for Wnt5a in regulating gene expression in hippocampal neurons. We thus asked whether Wnt5a deletion influences the levels of pre- and post-synaptic proteins. In *Nestin-Wnt5a*$^{fl/fl}$ mice at 1.5 months of age, immunoblotting analyses of hippocampal homogenates showed no differences in levels

of VGLUT1, synaptophysin, synapsin1, AMPA-type glutamate receptor subunits, GluA1 and GluA2, protein kinases CaMKIIα□□CaMKIIβ□□Akt, Erk, and the scaffold protein, PSD95, compared to control *Wnt5a*$^{fl/fl}$ mice (Figures 5.11 A and B). However, interestingly, we noticed a pronounced down-regulation (33% decrease) in the protein levels of GluN1, the essential NMDA receptor subunit, in mutant homogenates (Figure 5.11 A and B). Functional NMDA receptors are heterotetramers consisting of two obligatory GluN1 and different GluN2 subunits incorporated in a developmental and cell type-specific manner (Seeburg, 1993). We did not observe any changes in the levels of other NMDA receptor subunits, GluN2a/2b, that are co-expressed with GluN1 in the mature hippocampus (Figure 5.11 A and B), although the protein expression and stability of these subunits have been reported to be dependent on functional GluN1 subunits (Fukaya et al., 2003; Ultanir et al., 2007). This is likely because the GluN1 depletion in our study is less robust compared to that in *GluN1* null mice (Fukaya et al., 2003; Ultanir et al., 2007). In *CaMKII-Wnt5a*$^{fl/fl}$ mice at 3 months of age, we found a similar reduction in GluN1 levels, without any changes in GluN2a, GluN2b, GluA1 and GluA2 in hippocampal homogenates (Figure 5.12 A and B).

We assessed *GluN1* transcript levels using qPCR and observed a significant decrease (25%) in the 1.5 month *Nestin-Wnt5a*$^{fl/fl}$ hippocampus and (40%) in the 3 month *CaMKII-Wnt5a*$^{fl/fl}$ hippocampus, whereas the *GluN2a* and *GluN2b* transcripts were unaltered (Figures 5.11 C and 5.12 C). Given the decreased CREB phosphorylation with Wnt5a deletion, we asked whether Wnt5a regulates *GluN1* transcription in a CREB-dependent manner. We analyzed the mouse *GluN1* promoter region using a bioinformatics search (http://natural.salk.edu/CREB/) and identified three putative *CRE* sites (*CRE1* at -212 bp,

*CRE2* at -238 bp and *CRE3* at -770 bp) in the 1 kb region upstream of the transcription start site (Figure 5.13 A). In a dual luciferase assay, hippocampal neurons were co-transfected with vectors encoding for *Cypridina* luciferase driven by the *CRE*-harboring *GluN1* promoter region and *Gaussia* luciferase driven by the SV40 promoter as control. Stimulation of hippocampal neurons with Wnt5a conditioned media for 6 hr significantly increased *Cypridina* luciferase activity compared to control media (Figure 5.13 B). However, mutation of all three *CRE* sites in the *GluN1* promoter fragment abolished Wnt5a-induced luciferase activity (Figure 5.13 B). Further deletion analyses to delineate the responsible *CRE* elements revealed that the two proximal *CRE* elements (-212 to -216 bp and -238 to -242 bp) but not the distal *CRE3* site (-770 to -774) were necessary for Wnt5a-induced *GluN1* gene transcription (Figure 5.13 B). Together, these results reveal an unanticipated role for Wnt5a in enhancing *GluN1* transcription through a noncanonical Wnt pathway that involves calcium-CaMKII-CREB activation.

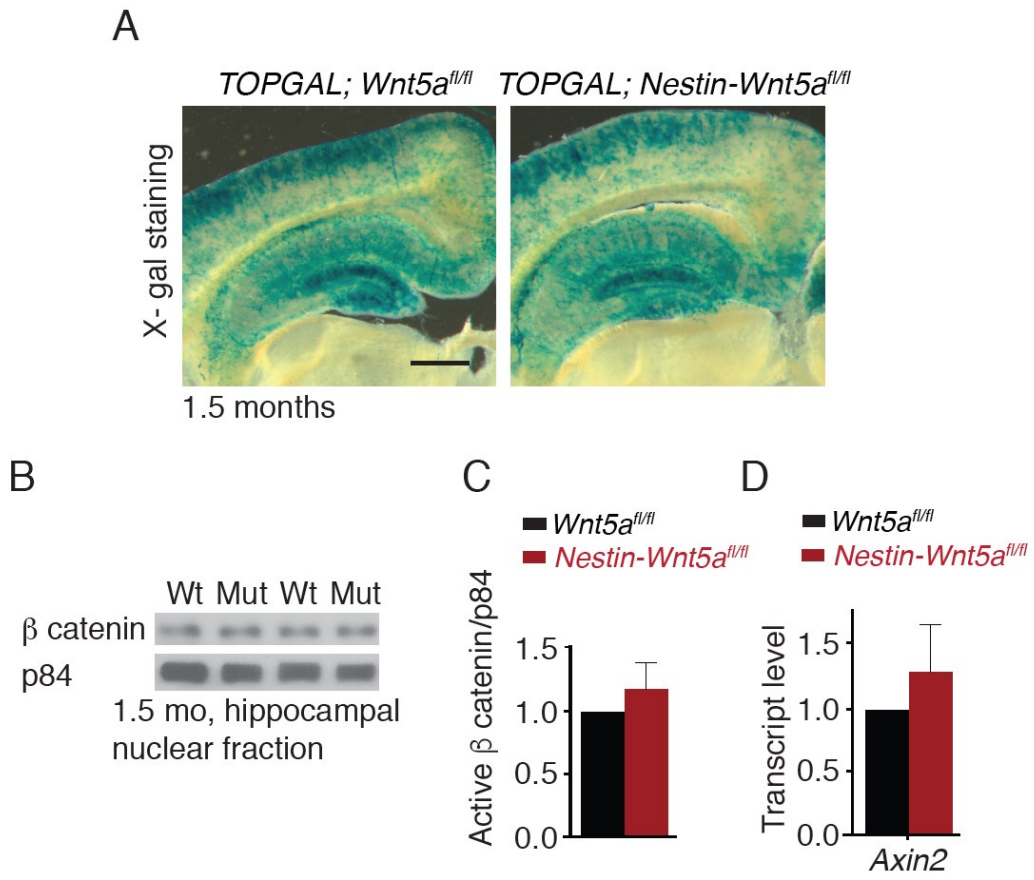
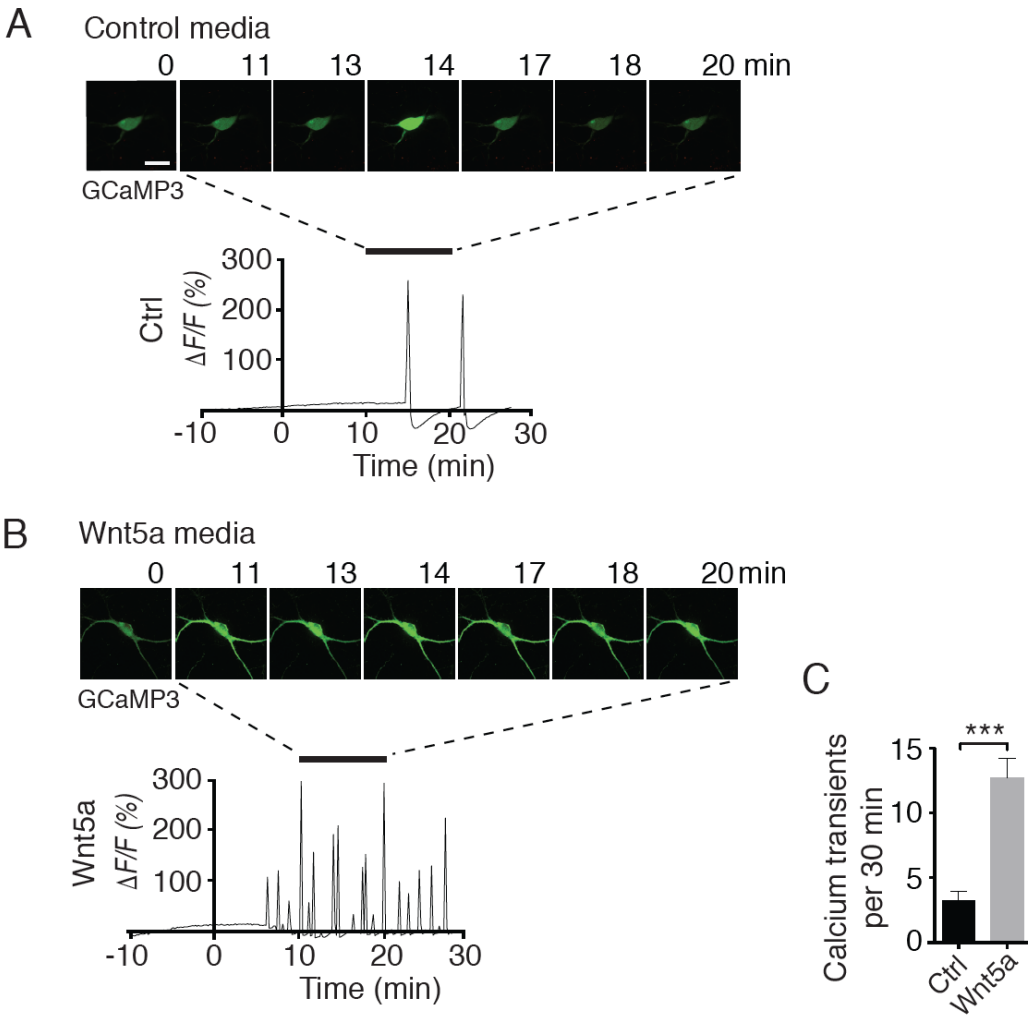



**Figure 5.1: Lost of *wnt5a* does not disturb β-catenin dependent pathway in 1.5 month *Nestin-Wnt5a*$^{fl/fl}$ hippocampus.**

**(A)** *Wnt5a* depletion does not affect β-catenin-mediated transcriptional activity in the hippocampus. X-gal staining of coronal brain sections shows comparable signal intensities between *TOPGAL;Nestin-Wnt5a*$^{fl/fl}$ mice and control litter-mates at 1.5 months. Scale bar: 400 □m. **(B, C)** Similar levels of nuclear (active) β-catenin in hippocampal nuclear fractions from 1.5 month *Nestin-Wnt5a*$^{fl/fl}$ mice and control litter-mates. The β-catenin immunoblot was reprobed for p84, a nuclear protein, for normalization. Results are means ± SEM from 11 mice per genotype. **(D)** *Axin2* transcript levels are unaffected by Wnt5a deletion in *Nestin-Wnt5a*$^{fl/fl}$ hippocampus. Results are means ± SEM from 6 mice per genotype.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GCaMP3
Ctrl
ΔF/F (%)
300
200
100
-10 0 10 20 30
Time (min)
B
Wnt5a media
0 11 13 14 17 18 20 min
GCaMP3
Wnt5a
ΔF/F (%)
300
200
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-10 0 10 20 30
Time (min)
C
Calcium transients per 30 min
15
10
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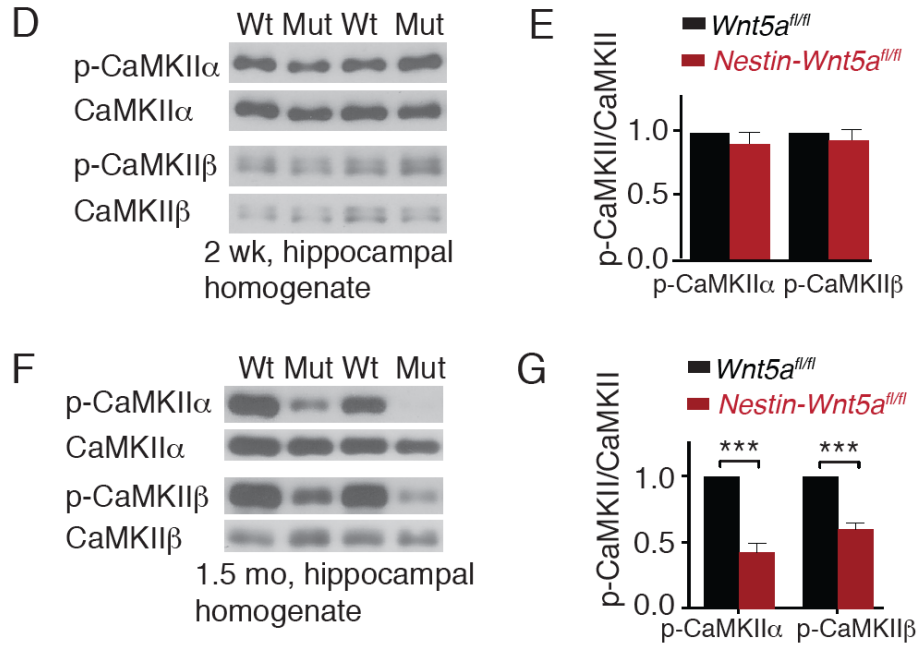
Ctrl
Wnt5a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CaMKIIα
p-CaMKIIβ
CaMKIIβ
2 wk, hippocampal homogenate
E
Wnt5a fl/fl
Nestin-Wnt5a fl/fl
p-CaMKII/CaMKII
1.0
0.5
0.0
p-CaMKIIα p-CaMKIIβ
F
Wt Mut Wt Mut
p-CaMKIIα
CaMKIIα
p-CaMKIIβ
CaMKIIβ
1.5 mo, hippocampal homogenate
G
Wnt5a fl/fl
Nestin-Wnt5a fl/fl
p-CaMKII/CaMKII
***
***
1.0
0.5
0.0
p-CaMKIIα p-CaMKIIβ

**Figure 5.2: The key component of calcium-CaMKII phosphorylation is decreased in 1.5 month *Nestin-Wnt5a$^{fl/fl}$* hippocampus.**

**(A, B)** Wnt5a treatment elicits a robust increase in calcium transients in hippocampal neurons. Rat hippocampal neuron cultures were transfected with GCaMP3 and treated with control L- or Wnt5a-conditioned media for 30 min. Scale bar: 20□m. Traces of calcium transients shown in below. **(C)** Wnt5a elicits a 5-fold increase in calcium transients compared to control media. Results are mean ± SEM from n=3 independent experiments (total of n=31 L media-treated cells and n=26 Wnt5a-treated neurons), ***p<0.001, unpaired two-tailed *t* test. (**D, E)** CaMKII phosphorylation is unaffected in the *Nestin-Wnt5a$^{fl/fl}$* hippocampus during development (2 weeks). Results are means ± SEM from n=9 mice per genotype. **(F, G)** Phosphorylation of CaMKII-α/β are significantly reduced in hippocampal homogenates from *Nestin-Wnt5a$^{fl/fl}$* (Mut) compared to *Wnt5a$^{fl/fl}$* litter-mates (Wt) at 1.5 months of age. Immunoblots were stripped and reprobed for total CaMKII-α and β for normalization. Results are mean ± SEM from n=6 mice per genotype, ***p<0.001, unpaired two-tailed *t* test.

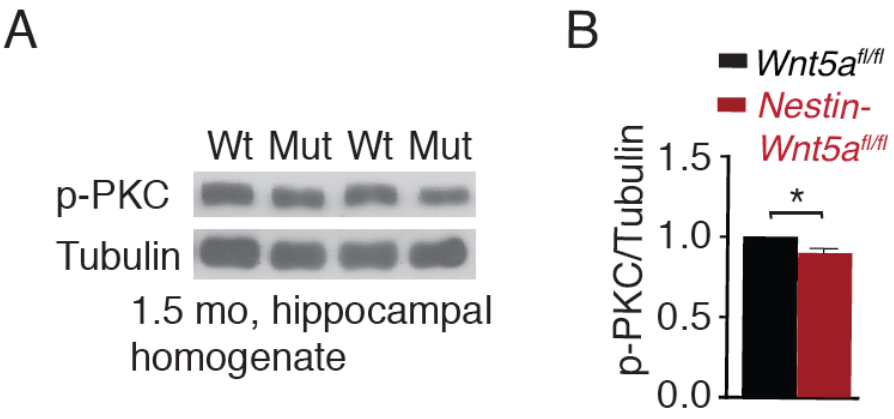


**Figure 5.3: Phosphorylation of protein kinase C (PKC) is slightly decreased in 1.5 month *Nestin-Wnt5a$^{fl/fl}$* hippocampus.**

(**A, B**) PKC phosphorylation is modestly decreased in 1.5 month *Nestin-Wnt5a$^{fl/fl}$* hippocampal homogenates. Results are means ± SEM from n=9 mice per genotype**,** *p<0.05, unpaired two-tailed *t* test.

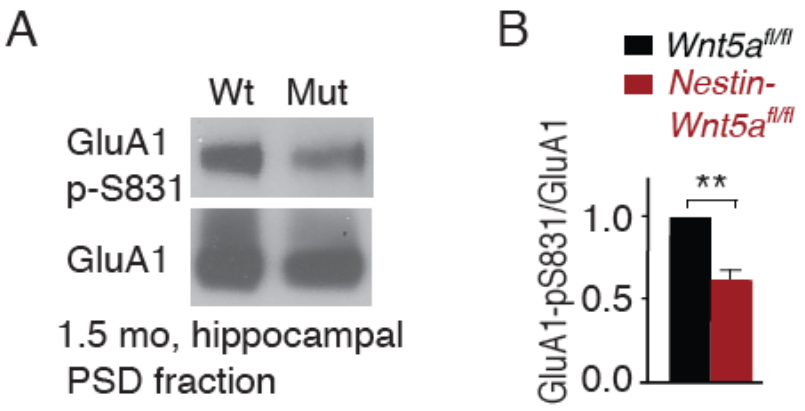


**Figure 5.4: Phosphorylation of GluA1 is decreased in 1.5 month *Nestin-Wnt5a*$^{fl/fl}$ hippocampal post-synaptic density fractions.**

**(A, B)** Phosphorylation of GluA1$^{Ser831}$ is significantly reduced in hippocampal post-synaptic density (PSD) fractions isolated from *Nestin-Wnt5a*$^{fl/fl}$ mice. Immunoblots were stripped and reprobed for total GluA1 for normalization. Results are means ± SEM from n=8 mice per genotype**,** **p<0.01, unpaired, two-tailed *t* test.

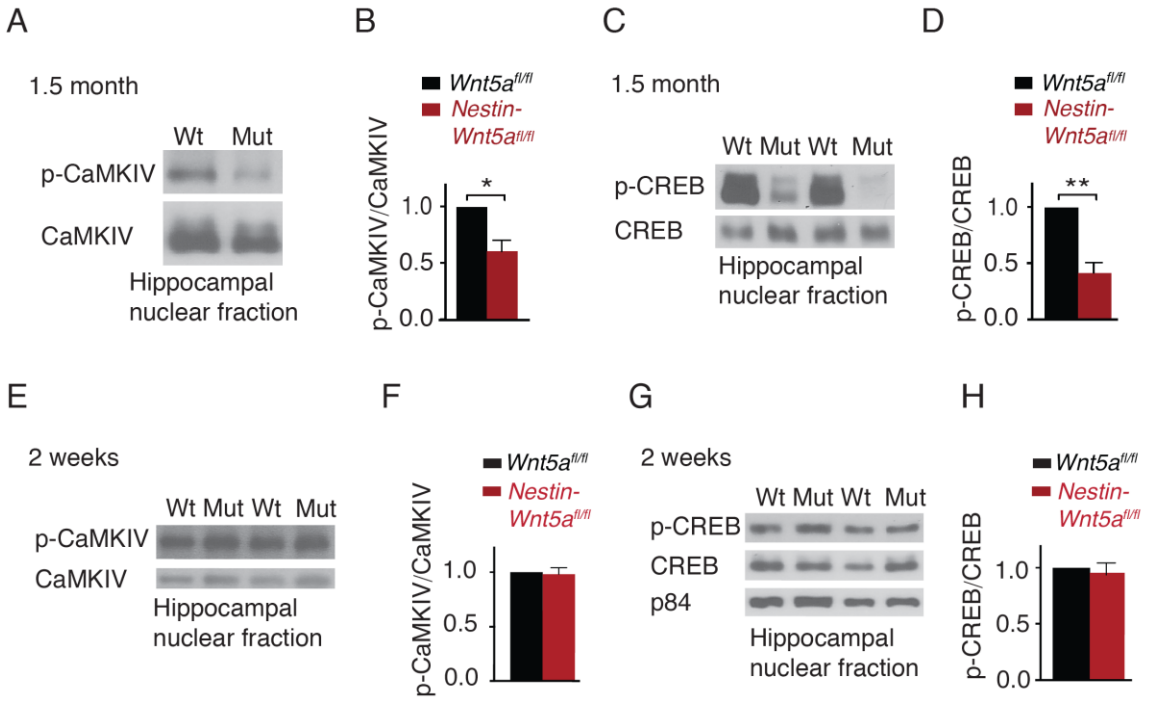


**Figure 5.5: Both CaMKIV and CREB activity are attenuated in 1.5 month *Nestin-Wnt5a$^{fl/fl}$* hippocampus nuclear fraction, but remain normal in 2 weeks *Nestin-Wnt5a$^{fl/fl}$* hippocampus nuclear fraction.**

**(A, B)** Phospho-CaMKIV levels are decreased in hippocampal nuclear fractions from 1.5 month old *Nestin-Wnt5a$^{fl/fl}$* mice. Immunoblots were reprobed for total CaMKIV for normalization. Results are mean ± SEM from n=6 mice per genotype, *p<0.05, unpaired two-tailed *t* test. **(C, D)** Phosphorylation of CREB is attenuated in *Nestin-Wnt5a$^{fl/fl}$* hippocampal nuclear fractions. Blots were reprobed for total CREB for normalization. Results are mean ± SEM from n=6 mice per genotype, **p<0.001, unpaired two-tailed *t* test. **(E, F)** CaMKIV phosphorylation is not altered in 2 week old *Nestin-Wnt5a$^{fl/fl}$* hippocampal nuclear fractions. Results are means ± SEM from n=6 mice per genotype. **(G, H)** CREB phosphorylation is normal in 2 week old *Nestin-Wnt5a$^{fl/fl}$* hippocampus. Results are means ± SEM from n=9 mice per genotype.

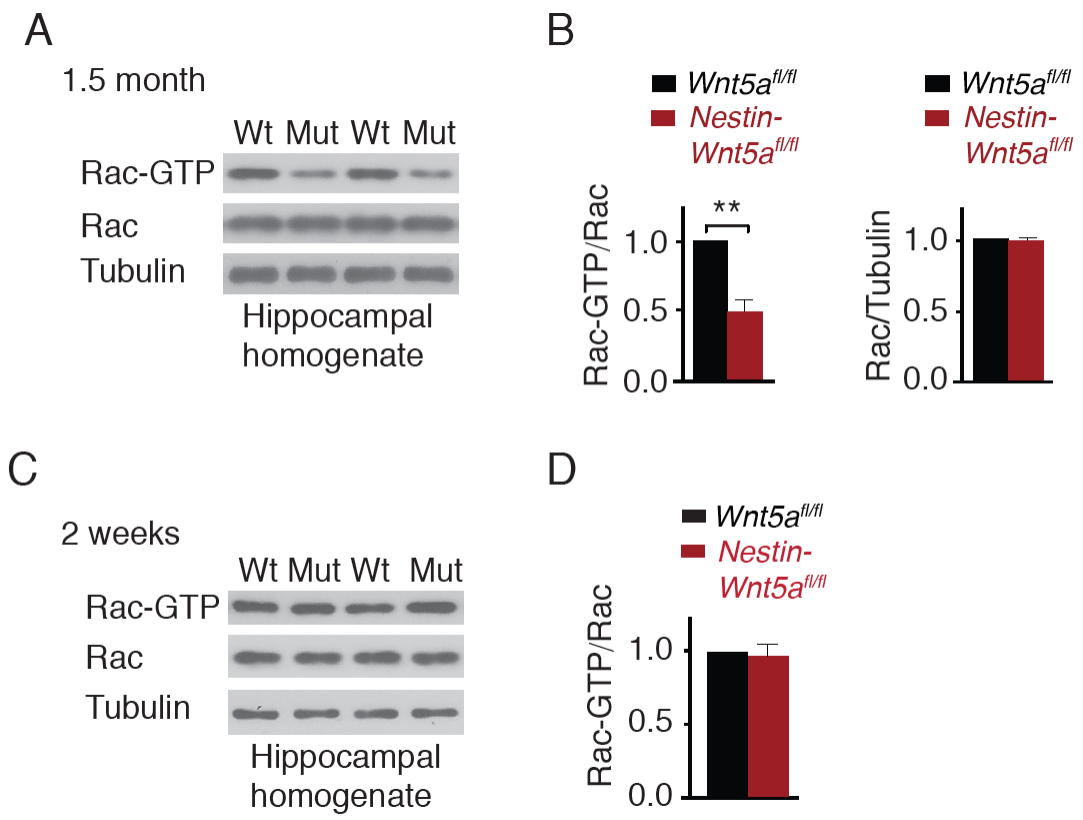


**Figure 5.6: Rac activity is attenuated in 1.5 month *Nestin-Wnt5a*$^{fl/fl}$ hippocampus but remain normal in 2 weeks *Nestin-Wnt5a*$^{fl/fl}$ hippocampus.**

**(A, B)** Rac1-GTP levels are reduced in hippocampal homogenates from 1.5month old *Nestin-Wnt5a*$^{fl/fl}$ mice. The total Rac1 level is not affected. Hippocampal homogenates were subjected to GST-PAK-PBD-agarose pull-downs and immunoblotted for Rac1 to assay for levels of Rac1-GTP. Immunoblots were stripped and reprobed for Rac1 and tubulin. Rac1-GTP signal intensities were normalized to total Rac1, while Rac1 was normalized to tubulin. Results are mean ± SEM from n=5 mice per genotype, **p<0.01, unpaired two-tailed *t* test. **(C, D)** There are no significant differences in Rac1-GTP levels between the *Nestin-Wnt5a*$^{fl/fl}$ and control hippocampus at 2 weeks. Results are means ± SEM from n=5 mice per genotype.

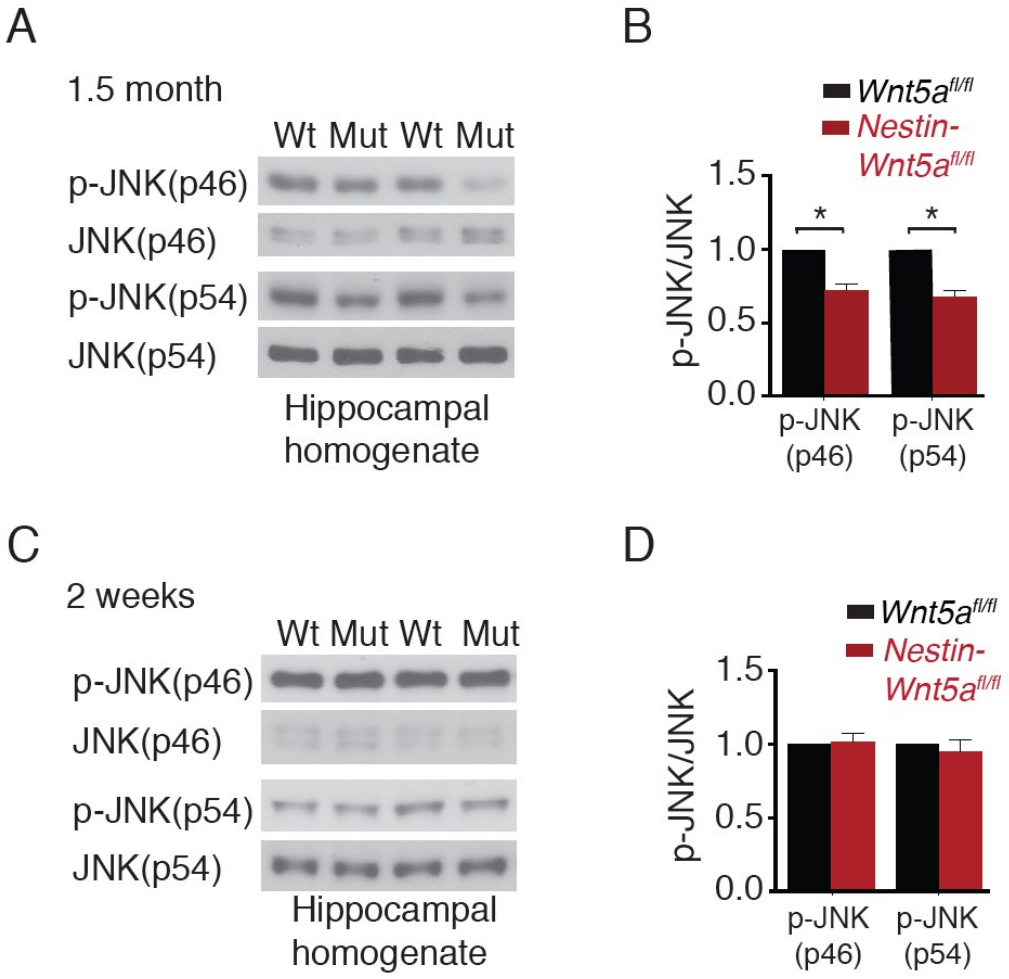


**Figure 5.7: Phosphorylation of JNK reduced in 1.5 month *Nestin-Wnt5a$^{fl/fl}$* hippocampus but not at 2 weeks *Nestin-Wnt5a$^{fl/fl}$* hippocampus.**

**(A, B)** Phospho-JNK levels are significantly decreased in hippocampal homogenates from *Nestin-Wnt5a$^{fl/fl}$* mice at 1.5 months. Results are means ± SEM from n=9 mice per genotype, *p<0.05, unpaired two-tailed *t* test. **(C, D)** There are no significant differences in JNK phosphorylation levels between the *Nestin-Wnt5a$^{fl/fl}$* and control hippocampus at 2 weeks. Results are means ± SEM from n=5 mice per genotype.

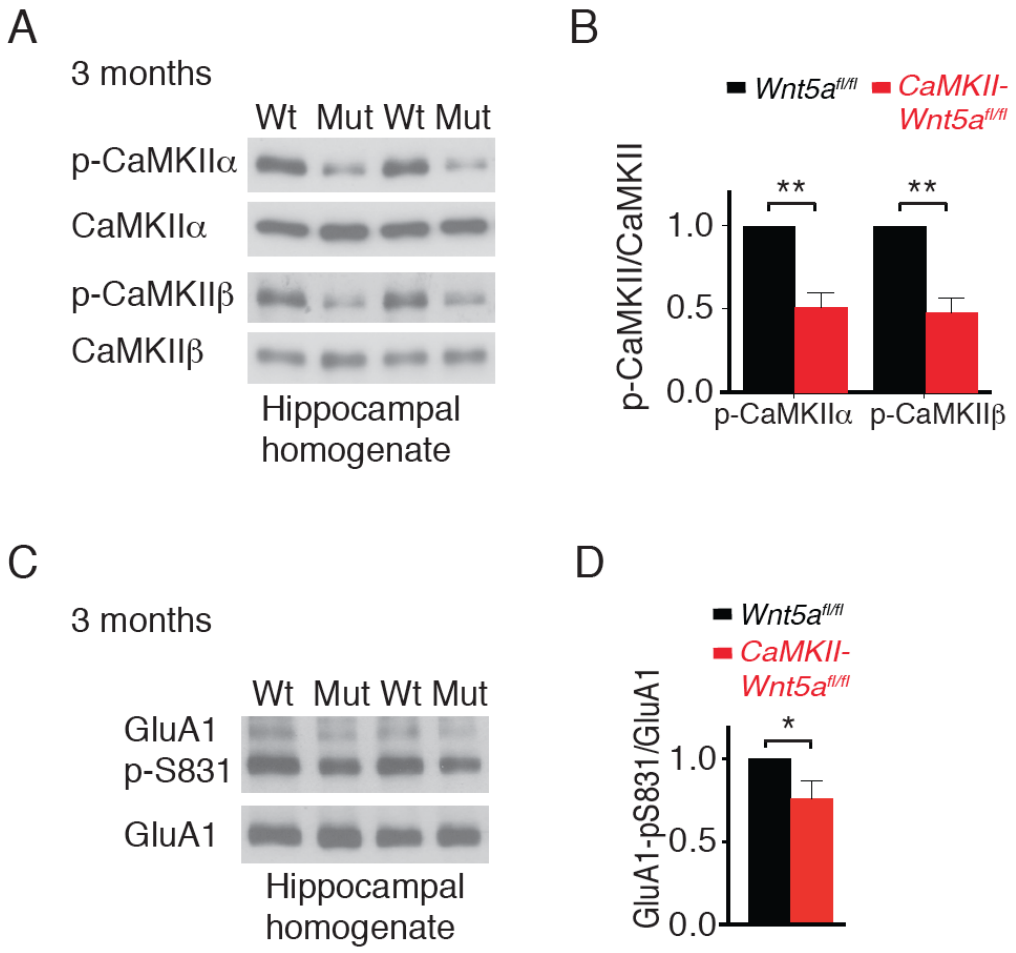


**Figure 5.8 Attenuated phosphorylation of CaMKII and GluA1 in the mature *CaMKII-Wnt5a$^{fl/fl}$* hippocampus.**

**(A, B)** Phospho-CaMKII levels are markedly decreased in 3 month old *CaMKII-Wnt5a$^{fl/fl}$* hippocampus. Results are means ± SEM from n=6 mice per genotype, **p<0.01, unpaired two-tailed *t* test. **(C, D)** Reduced GluA1$^{Ser831}$ phosphorylation levels in 3 month old *CaMKII-Wnt5a$^{fl/fl}$* hippocampus. Results are means ± SEM from n=6 mice per genotype, *p<0.05, unpaired two-tailed *t* test.

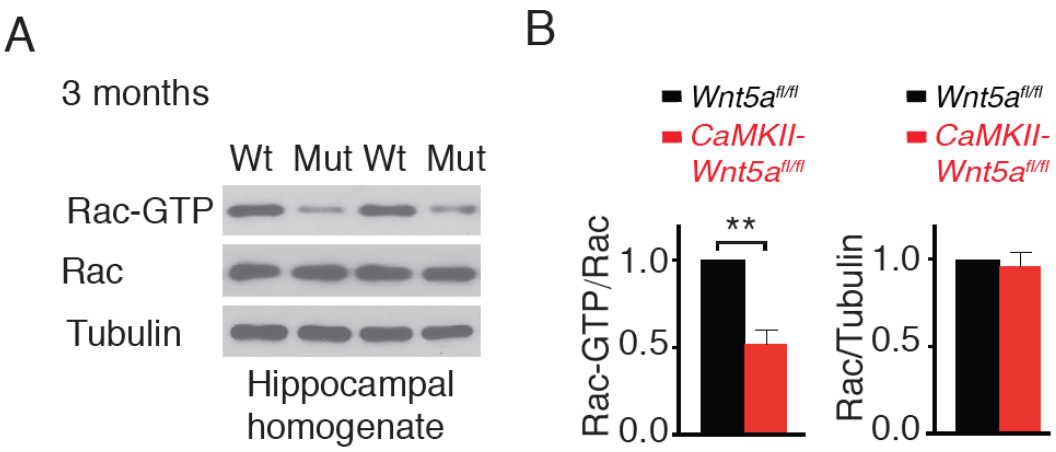


**Figure 5.9 Attenuated Rac activity in the mature *CaMKII-Wnt5a*$^{fl/fl}$ hippocampus.**

(**A, B**) A pronounced decrease in Rac1 activity in hippocampal homogenates from 3-month old *CaMKII-Wnt5a*$^{fl/fl}$ mice. The total Rac1 protein level is not affected. Results are means ± SEM from n=6 mice per genotype, **p<0.01, unpaired two-tailed *t* test.

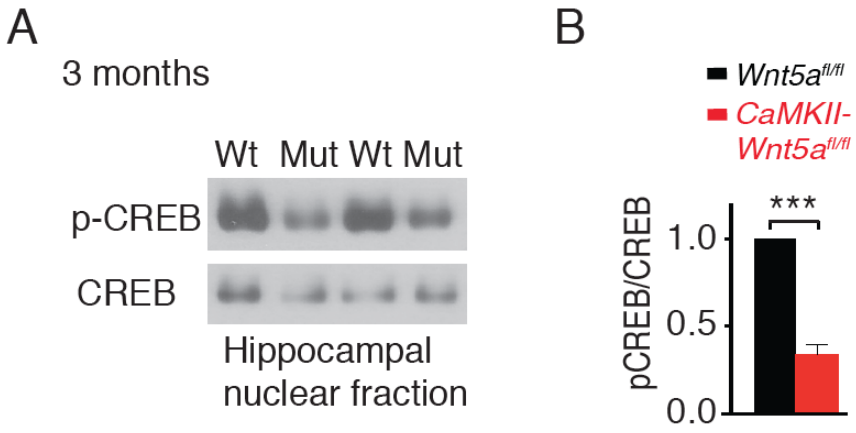


**Figure 5.10 CREB phosphorylation decreased in the 3 month old *CaMKII-Wnt5a$^{fl/fl}$* hippocampus.**

(A, B) Down-regulation of phosphorylated CREB in 3 month old *CaMKII-Wnt5a$^{fl/fl}$* hippocampal nuclear fractions. Results are means ± SEM from n=5 mice per genotype, ***p<0.001, unpaired two-tailed *t* test.

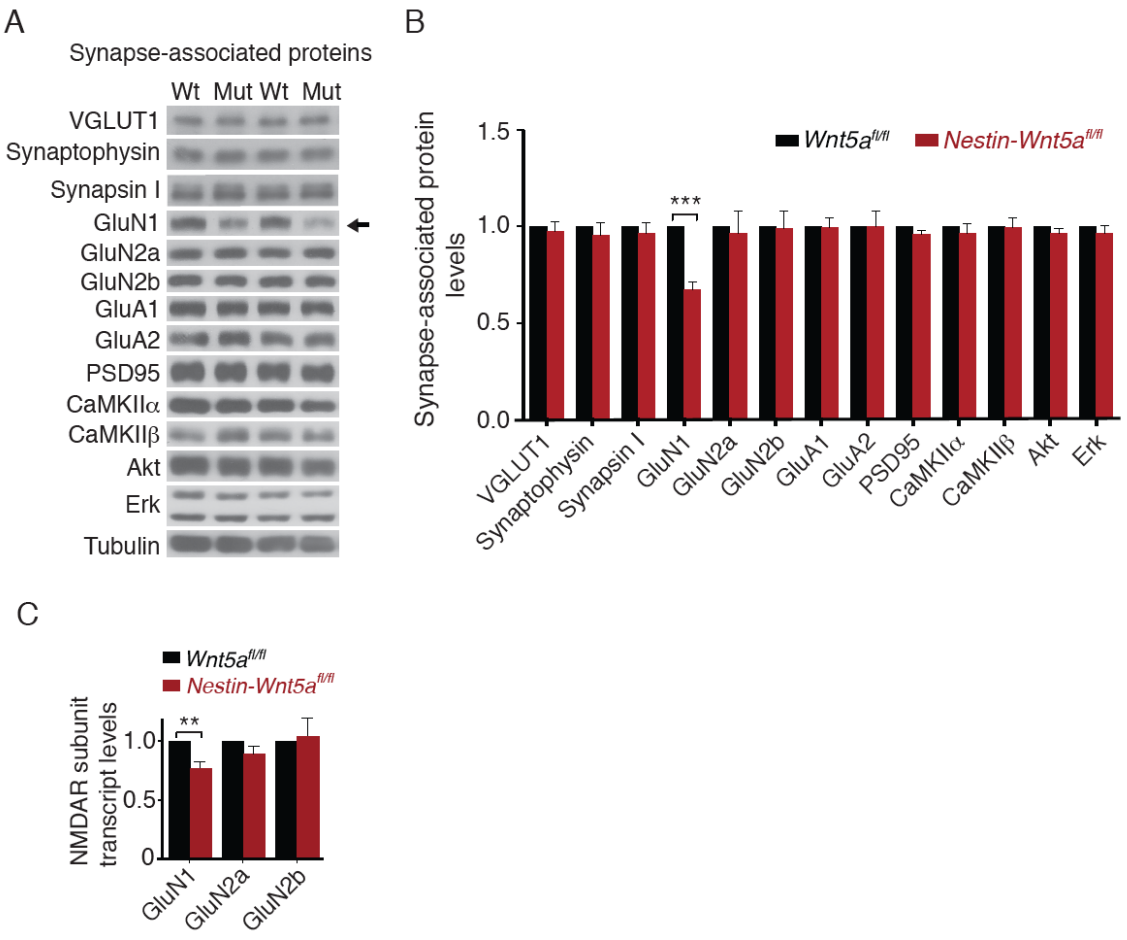


**Figure 5.11: *GluN1* protein and transcript level are decreased in *Nestin-Wnt5a*$^{fl/fl}$ hippocampus.**

**(A, B)** Representative immunoblots of synapse-associated signaling and structural proteins. Decreased protein levels of the obligatory NMDA receptor subunit, GluN1, in hippocampal homogenates from 1.5-month *Nestin-Wnt5a*$^{fl/fl}$ mice (arrow). Immunoblots were stripped and reprobed for tubulin for normalization. **(B)** Densitometric quantification of immunoblots of synaptic proteins in hippocampal homogenates from 1.5 month old *Nestin-Wnt5a*$^{fl/fl}$ and control mice. Densitometric signal intensities for each protein were normalized to tubulin, and values expressed relative to control homogenates. Results are mean ± SEM from n=6 mice per genotype, ***p<0.001, unpaired two-tailed *t*

test. **(C)** Quantitative real-time PCR analysis shows a decrease in *GluN1* transcript levels but not of *GluN2a/b* in the *Nestin-Wnt5a*$^{fl/fl}$ hippocampus. Results are mean ± SEM from n=6 mice per genotype, **p<0.01, unpaired two-tailed *t* test.

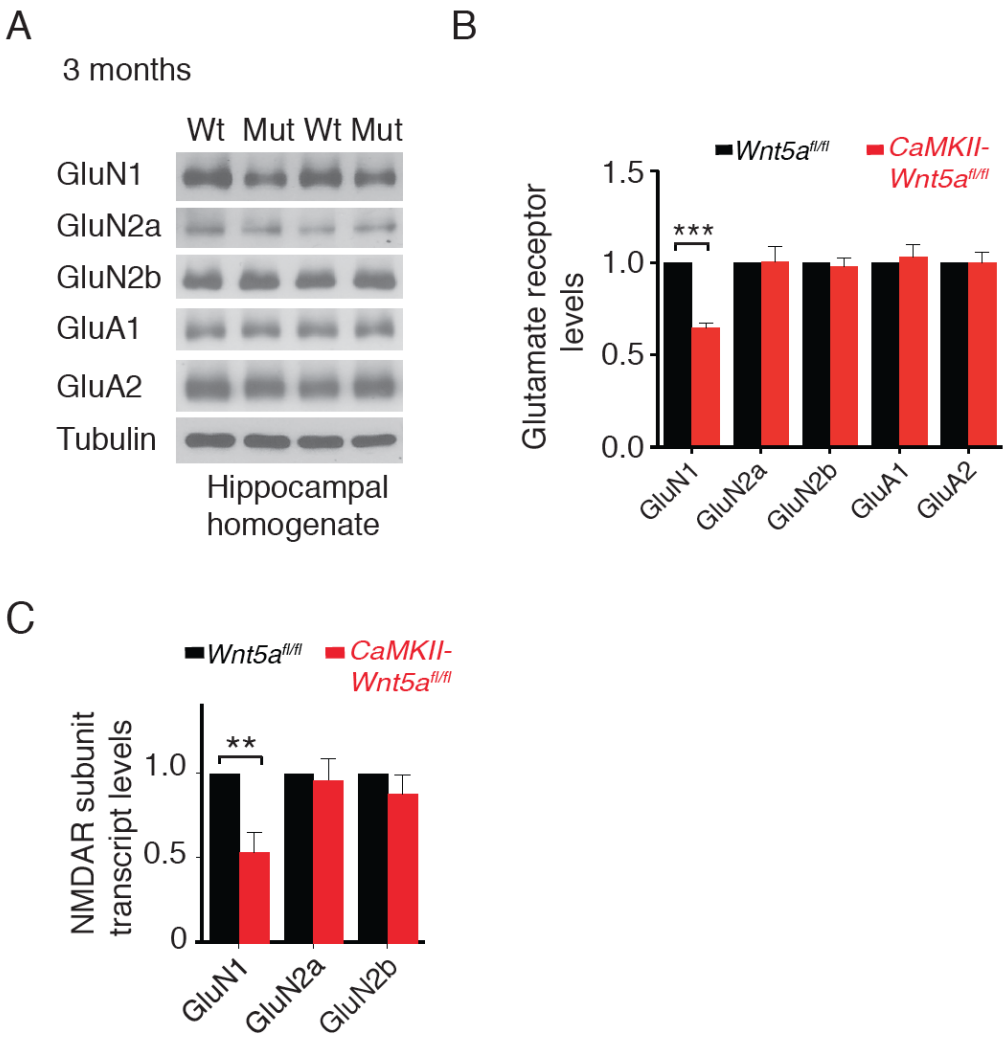


**Figure 5.12: *GluN1* protein and transcript level are decreased in mature *CaMKII-Wnt5a$^{fl/fl}$* hippocampus**

(**A, B**) GluN1 protein levels are decreased in 3 month old *CaMKII-Wnt5a$^{fl/fl}$* hippocampal homogenates. Other glutamate receptor subunits are unaffected. Results are mean ± SEM from 6 mice per genotype, ***p<0.001, unpaired two-tailed *t* test. **C)** Quantitative real-time PCR analysis shows a decrease in *GluN1* transcript levels but not of *GluN2a/b* in the *CaMKII-Wnt5a$^{fl/fl}$* hippocampus. Results are mean ± SEM from n=6 mice per genotype, **p<0.01, unpaired two-tailed *t* test.

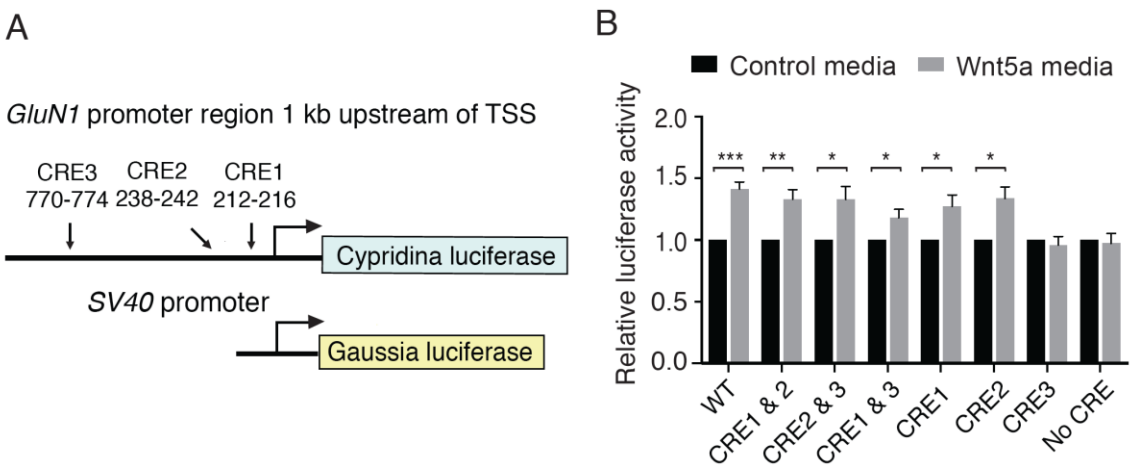


**Figure 5.13: Proximal CRE1 and CRE2 sites in *GluN1* promoter are important for Wnt5a mediated *GluN1* transcription.**

**(A)** A 1kb region upstream of the mouse *GluN1* promoter harbors three putative *CRE* sites. Hippocampal neurons were co-transfected with luciferase reporter constructs, *Cypridina* luciferase driven by *GluN1* promoter region, and *Gaussia* luciferase driven by SV40 promoter as a control. Hippocampal neurons were treated with control or Wnt5a media for 6 hr and then harvested to measure luciferase activity. **(B)** Structure and fnuction analysis of all three *CRE* sites on *GluN1* promoter by dual luciferase assy. Wnt5a promotes a significant increase in *GluN1* promoter driven luciferase activity. The two proximal binding sites (*CRE1 and CRE2*) is sufficient for the Wnt5a mediated increase in luciferase activity, however the distal *CRE* site (*CRE3*) showed no function in inducing luciferase activity. Results are mean ± SEM for n=6 independent experiments, **p<0.01, ***p<0.001, unpaired two-tailed *t* test.

## Discussion

A vast body of literature supports the notion that Wnt5a signaling work through non-canonical pathways during development. To investigate the signaling pathways by which Wnt5a influences morphological changes and animal learning and memory behavior in mature mice, we first utilized *TOPGAL;Nestin-Wnt5a*$^{fl/fl}$ mice to monitor the canonical β-catenin transcriptional ability with *Laz reporter*. The *Laz* level are comparable in abult mutant mice. We further checked the active form of β-catenin, the downstream transcription factor in the canonical pathway, prepared from the nuclear fraction and did not obserse a change between 1.5 month-old WT and Wnt5a mutant mice. Additionally, the transcript of *Axin2,* a β-catenin target gene, also showed a normal level in mutant mice compared to their WT litermate. These results together suggested that Wnt5a signaling in the adult hippocampus does't activate canonical pathway. To test whether Wnt5a signaling work through non-canonical pathways in the hippocampus, we employed phospho-specific antibodies to examine the activation of Wnt5a downstream effectors, including CaMKII, PKC and JNK, from hippocampal lysates prepared from 1.5 month old *Nestin-Wnt5a*$^{fl/fl}$ and 3 months old *CaMKII-Wnt5a*$^{fl/fl}$ mutant mice. Interestingly, We found that the phosphorylation of CaMKII-α and CaMKII -β were significantly attenuated in hippocampal lysates. This evidence prompt us further test levels of CaMKIV and CREB from the nuclear fraction of mature hippocampal neurons. Indeed, both CaMKIV and CREB phosphorylation are attenuated in the mutant mice. Using biochemical screen for proteins important for neuronal structure and transmission, surprisingly, we found that GluN1, the major NMDA subunit is reduced in the mature hippocampus. The reduce of GluN1 protein level in 3 month old *CaMKII-Wnt5a*$^{fl/fl}$ mice

might account for the deficits of induction period of NMDA receptor dependent LTP (Figure 3.3). Together, these results reveal an unanticipated role for Wnt5a in enhancing *GluN1* transcription in hippocampus through a non-canonical Wnt pathway that involves calcium-CaMKII-CREB activation.

Given the dramatic alterations that we observed in dendritic complexity in Wnt5a mutant neurons, we reason that Wnt5a might regulate actin dynamics in hippocampal dendrites, necessary for growth and maintenance of dendrites and spines. Previous studies have shown that the small GTPases Rho, Rac and Cdc42 are critical regulators of actin assembly. Thus, we perform pull-down assays to probe for active GTPases in hippocampal lysates prepared from 1.5 month old *Nestin-Wnt5a*$^{fl/fl}$ and 3 months old *CaMKII-Wnt5a*$^{fl/fl}$ mice and their wild-type littermates. The active form of Rac-GTP is significantly reduced in the mature hippocampal neurons.

Here we identified that Wnt5a function through the non-canonical pathway in the mature hippocampus. The downstream signaling effectors, including CaMKII, Rac1, and CREB, known to be important for dendrite structure development and maturation (Wong and Ghosh, 2002) were reduced as early as 1.5 months of age in *Nestin-Wnt5a*$^{fl/fl}$ mice. The down-regulation of small GTPase-mediated signaling and reduced of excitatory synaptic transmission are also critical factors in stabilizing neuronal connectivity (Cline and Haas, 2008; Nakayama et al., 2000; Van Aelst and Cline, 2004).

## Methods

### Animals

All procedures relating to animal care and treatment conformed to institutional and NIH guidelines. Animals were housed in a standard 12:12 light-dark cycle. The generation of *Wnt5a*$^{fl/fl}$ mice has been previously described (Ryu et al., 2013). *Wnt5a*$^{fl/fl}$ mice were backcrossed to *C57BL/6* background for at least 7 generations and maintained on a *C57BL/6* background. *Nestin-Cre* and *TOPGAL* mice were obtained from Jackson Laboratory and *CaMKII-Cre* mice (T29-1 line) were a generous gift from Dr. Nicholas Gaiano. Sprague Dawley rats were purchased from Charles River. Hippocampal neuron cultures were established from embryonic day 18 (E18) rat pups as previously described (Araki et al., 2015).

### Reagents and antibodies

The antibodies used in this study were previously validated for the following applications: Wnt5a (R&D Systems; AF645, western bloting), VGLUT1 (Millipore; AB5905, western blotting), PSD95 (NeuroMab, western blotting), GluA1 (JHMI monoclonal antibody core, western blotting), GluA2 (JHMI monoclonal antibody core, western blotting), GluN1 (Millipore; MAB363, western blotting), CaMKII (Cell Signaling; 4436, western blotting), Arc (JHMI monoclonal antibody core, western blotting), Tubulin (Sigma Aldrich; T-6199, western blotting), Rac1 (Millipore; 05-389, western blotting), P-CaMKII (Thr286) (Cell Signaling; 3316 and 12716, western blotting), P-CaMKIV (Thr196) (Santa Cruz; sc-28443-R, western blotting), CaMKIV

(Abcam; ab3557, western blotting), P-CREB (Ser133) (Cell Signaling; 9198, western blotting), CREB (Cell Signaling; 9104, western blotting), GluN2a (JHMI monoclonal antibody core, western blotting), GluN2b (JHMI monoclonal antibody core, western blotting), Akt (Cell Signaling; 9272, western blotting), Erk (Cell Signaling; 9102, western blotting), Nuclear matrix protein p84 (GeneTex; GTX70220, western blotting), Active β-catenin (Millipore; 05-665, western blotting), p-JNK (Cell Signaling; 9251, western blotting), JNK (Cell Signaling; 9252, western blotting), pan-p-PKC (Cell Signaling; 9371, western blotting), p-GluA1-Ser831 (Millipore; AB5847, western blotting), NeuN (Millipore; MAB377, immuno-histochemistry), Neurofilament (Millipore; AB5539, immunohistochemistry), MAP2 (Sigma Aldrich; M9942, immunohistochemistry) Synaptophysin (Sigma Aldrich; S5768, western blotting) and Synapsin 1 (BD Transduction laboratories, 611392, western blotting). Golgi-Cox staining was done using the FD Rapid GolgiStain$^{TM}$ kit (FD Neurotechnologies Inc, PK401). Rac1/Cdc42 GTPase activity was measured by a kit (EMD Millipore; 17-441). *GluN1* promoter activities were followed with BioLux *Cypridina* and *Gaussia* luciferase assay kits (New England Biolabs Inc, E3309S and E3300S). Wnt5a conditioned media and L-cell conditioned media were harvested from Wnt5a producing cells (ATCC CRL-2814) and L cells (ATCC CRL-2648). Both cell lines were first cultured in DMEM supplemented with 10% FBS for 2 days, and then the medium was changed to Neurobasal medium supplemented with B27 for 24 hr.

**X-gal staining**

Brains isolated from mice anesthetized by isoflurane were quickly sectioned using a vibratome as described below in slice preparations. Brain slices (200 μm) were then post-fixed in 0.4% PFA for 15 min, incubated in Buffer B (100mM phosphate buffer at pH 7.4, 2mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% IGEPAL) then stained in Buffer B plus 5mM potassium ferricyanide, 5mM potassium ferrocyanide, and 1mg/ml X-gal for 4 days. Reactions were stopped with 4% PFA and then washed with TBS and mounted in VectaShield (Vector Laboratories).

**Rac1 GTPase activity**

The Rac1/Cdc42 Activation Assay (EMD Millipore; 17-441) was used to detect Rac1 activity in hippocampal homogenates. Briefly, hippocampal lysates were pre-cleared with glutathione-agarose beads (100 μl). Supernatants were incubated with a fusion protein (GST-PAK) coupled to agarose beads. GST-PAK specifically binds to Rac1 or Cdc42 in its GTP-bound form. Rac1-GTP in the pull-downs was detected by immunoblotting using a Rac1-specific antibody.

**Calcium imaging**

Hippocampal neurons from E18 rat pups were plated onto glass cover-slips coated with poly-L-lysine (1 μg/ml; Sigma-Aldrich) and grown in Neurobasal growth medium supplemented with 2% B27, 2 mM GlutaMax, 50 U/mL penicillin, 50 μg/mL streptomycin, and 5% FBS. Neurons were switched to serum-free Neurobasal medium 24 hrs post-seeding and fed twice a week. Neurons were co-transfected with GCaMP3 and TdTomato at 14–15 days in vitro (d.i.v.) using lipofectamine 2000 (Invitrogen).

Briefly, coverslips containing neurons were assembled onto a closed perfusion chamber and continuously perfused with ACSF buffer with 2mM $Mg^{2+}$. After 10 min of baseline recording ($F_0$), neurons were perfused with Wnt5a- or L-conditioned media diluted (1:500) in ACSF buffer with 1mM $Mg^{2+}$ for 30 min. All imaging experiments were performed at 37°C using a Zeiss spinning disk confocal (Carl Zeiss, Thornwood, NY). The GCaMP3 was imaged at 488 nm excitation and collected through a 505–550 nm filter, while the TdTomato signal was imaged at 561 nm excitation and 575–615 nm emission. Neurons were imaged using a 63× oil objective (N.A. = 1.40) at a rate of 6 images per min. Images were analyzed using ImageJ software (NIH) by calculating the normalized change in average GCaMP3 over TdTomato fluorescence intensities from neuronal soma. The fluorescence intensity change is expressed as $\Delta F/F_o$ and the amplitude of fluorescence change ($\Delta F_{max}/F_o$) represents the extent of GCaMP3 intensity change.

**Statistical analyses**

All Student's *t* tests were performed using two-tailed, unpaired, and a confidence interval of 95%. One-way or two-way ANOVA analyses were performed when more than two groups were compared. Statistical analyses were based on at least 3 independent experiments, and described in the figure legends. All error bars represent the standard error of the mean (s.e.m).

# Chapter 6: Concluding remarks

In this study, we identify Wnt5a to be an essential factor for synaptic plasticity and long-term stability of the dendritic architecture in the adult hippocampus as well as for related cognitive functions. Wnts are evolutionarily conserved signaling molecules that have been classically associated with embryonic patterning and establishment of neural circuits (Budnik and Salinas, 2011; Ciani and Salinas, 2005; Logan and Nusse, 2004). However, the roles of these classical developmental cues in the adult brain have been less defined. We provide several advances in this study; First, although there are 19 vertebrate Wnts, our results provide evidence that deletion of a single member, Wnt5a, is sufficient to elicit profound disruptions in synaptic plasticity, structural maintenance, and learning and memory behaviors in adult animals, highlighting the importance of this particular non-canonical Wnt in later-life functions. Second, we show that neuronal Wnt5a is essential for the maintenance of dendritic arbors and spines in the adult CA1 hippocampus, although dispensable for their establishment during development. Third, we provide evidence that Wnt5a likely functions via CaMKII- and Rac1-mediated signaling cascades as well as by regulating the synthesis of GluN1 NMDA-type receptor subunits in mature hippocampal neurons. Finally, in the absence of Wnt5a, we observe molecular, physiological and behavioral changes that precede the onset of morphological defects by several months, suggesting that these early changes can be predictive of disease-related structural abnormalities in the adult brain.

The findings that the early deletion of Wnt5a in neurons did not elicit any structural abnormalities in CA1 pyramidal neurons in 1 month old *Nestin-Wnt5a*$^{fl/fl}$ mice suggests that neuronal Wnt5a is dispensable for the establishment or maturation of hippocampal

connectivity *in vivo*. These results were surprising in the context of reported developmental functions for Wnt5a in cultured hippocampal neurons, and in embryonic processes in other brain regions (Bian et al., 2015; Varela-Nallar et al., 2010a; Zhang et al., 2007). In hippocampal neurons, several signaling pathways have been shown to influence dendrite morphogenesis, maturation and stability *in vitro* and *in vivo* (Emoto, 2012; Koleske, 2013; Valnegri et al., 2015). Thus, in the absence of Wnt5a, other signaling mechanisms including other Wnt molecules (Rosso et al., 2005; Wayman et al., 2006), could provide trophic support to hippocampal CA1 dendrite arbors and spines at least for the first several months of life in mice. Alternatively, Wnt5a derived from non-neuronal sources may support hippocampal formation in the absence of neuron-derived Wnt5a. However, the profound defects in older *Nestin-Wnt5a*$^{fl/fl}$ and *CaMKII-Wnt5a*$^{fl/fl}$ mice suggest that these mechanisms are unable to compensate for Wnt5a loss at later stages of life. Rather, our findings argue that Wnt5a, likely derived from CA1 pyramidal neurons themselves, is critical for sustaining dendritic architecture in the adult hippocampus. To date, our limited understanding of the molecular cues that influence neuronal morphology in adult animals has largely come from analyses of cortical neurons in genetically modified mice. Among the examples are adult mice with deletion of BDNF and its receptor TrkB (Gorski et al., 2003; Xu et al., 2000), the adhesion molecule delta-catenin (Matter et al., 2009), and the tumor suppresor PTEN (Chow et al., 2009). Our findings identifying Wnt5a as being essential for the maintenance of adult CA1 hippocampal neurons is relevant to understanding the structural bases of hippocampus-dependent behaviors.

Recent studies indicate that exogenous application of Wnt proteins and broad-spectrum Wnt inhibitors affect synaptic plasticity and cognitive functions in adult organisms (Cerpa et al., 2011; Chen et al., 2006; Fortress et al., 2013; Jensen et al., 2012; Liu et al., 2014; Maguschak and Ressler, 2011; Oliva et al., 2013; Shimogori et al., 2004; Tabatadze et al., 2012; Vargas et al., 2014). Our results suggest that synaptic plasticity is most susceptible to the loss of Wnt5a. *CaMKII-Wnt5a*$^{fl/fl}$ mice had impaired hicampal CA1 LTP and related behavioral defects at 3 months of age, a time when basal synaptic transmission and dendritic morphology are intact. The normal pre-synaptic properties indicates that Wnt5a acts primarily at post-synaptic sites. Recombinant Wnt5a has previously been shown to acutely modulate NMDAR-mediated synaptic transmission in rat hippocampal slices (Cerpa et al., 2011). Our biochemical analyses suggest that Wnt5a may modify synaptic strength through CaMKII-mediated signaling events including the phosphorylation and subsequent trafficking/conductance of AMPA-type glutamate receptors as well as Rac1-dependent regulation of actin dynamics in dendritic spines. Our findings also suggest that attenuated NMDA receptor synthesis may be a contributing factor to the functional and behavioral defects in Wnt5a mutant mice. Mice with forebrain-specific deletion of *GluN1* using the *CaMKIIα-Cre* (T29-1) transgenic mice lacked NMDA-mediated currents and plasticity at CA1 synapses, and had profound defects in spatial memory acquisition (Tsien et al., 1996b). Although NMDA receptor-mediated LTD is impaired in mice with *CaMKII-Cre*-mediated deletion of *GluN1* (Tsien et al., 1996b), that we observed normal LTD responses in *CaMKII-Wnt5a*$^{fl/fl}$ mice can be attributed to the fact that residual GluN1 expression in Wnt5a mutant mice may still

allow sufficient $Ca^{2+}$ influx to promote LTD, consistent with the view of differential $Ca^{2+}$ requirements for LTD versus LTP (Dudek and Bear, 1992; Mulkey and Malenka, 1992).

Although molecular changes in key signaling cascades known to be critical for hippocampal circuitry are initiated early with Wnt5a deletion, intriguingly, there was a prolonged delay in the appearance of structural defects. Biochemical alterations in signaling effectors, CaMKII, Rac1, and CREB known to be critical for dendrite arbor morphogenesis and maturation (Wong and Ghosh, 2002), were detected as early as 1.5 months of age in *Nestin-Wnt5a*$^{fl/fl}$ mice, but degenerative changes in structure were not manifested until several months later in adult mice. We reason that neuronal architecture in the mature brain may be tolerant of a range of downregulation of the levels and activities of these signaling effectors, in contrast to developmental stages. The down-regulation of small GTPase-mediated signaling or excitatory synaptic transmission, postulated to be critical determinants in stabilizing neuronal connectivity (Cline and Haas, 2008; Nakayama et al., 2000; Van Aelst and Cline, 2004) may not be sufficient to trigger degenerative changes until a critical low level of activity is reached over time. Alternatively, there may be yet to be identified intracellular signaling activities that may be down-regulated at a slower pace that underlie the delayed appearance of structural abnormalities in Wnt5a mutant mice.

The phenotypes in adult Wnt5a mutant mice are reminiscent of the chronic neurodegenerative scenario. The early LTP defects and the cognitive decline followed by retraction of dendrites and spine loss bear similarities to the progression of events in animal models of Alzheimer's disease (AD) (Walsh and Selkoe, 2004), although we found no measurable neuronal loss in adult Wnt5a mutant mice. Additionally, the early

molecular changes observed in Wnt5a mutants may be predictive of disease-related structural abnormalities. Thus, early and modest insults to calcium- and cytoskeletal-based signaling cascades may accumulate over time to ultimately result in dendritic regression in neurodegenerative situations. Recent genetic evidence implicates deficiencies in Wnt signaling, largely the canonical arm, in the synaptic dysfunction and cognitive impairments in AD (De Ferrari and Inestrosa, 2000; De Ferrari et al., 2007). Our work highlights the significance of non-canonical Wnt signaling in maintaining synaptic function and connectivity in the adult brain and suggests a neuroprotective mechanism that can be potentially harnessed for therapeutic purposes in pathological situations.

## References:


Adler, P.N. (2002). Planar signaling and morphogenesis in Drosophila. Developmental cell *2*, 525-535.

Ahmad-Annuar, A., Ciani, L., Simeonidis, I., Herreros, J., Fredj, N.B., Rosso, S.B., Hall, A., Brickley, S., and Salinas, P.C. (2006). Signaling across the synapse: a role for Wnt and Dishevelled in presynaptic assembly and neurotransmitter release. The Journal of cell biology *174*, 127-139.

Anderton, B.H., Callahan, L., Coleman, P., Davies, P., Flood, D., Jicha, G.A., Ohm, T., and Weaver, C. (1998). Dendritic changes in Alzheimer's disease and factors that may underlie these changes. Progress in neurobiology *55*, 595-609.

Angers, S., and Moon, R.T. (2009). Proximal events in Wnt signal transduction. Nature reviews Molecular cell biology *10*, 468-477.

Anggono, V., and Huganir, R.L. (2012). Regulation of AMPA receptor trafficking and synaptic plasticity. Current opinion in neurobiology *22*, 461-469.

Araki, Y., Zeng, M., Zhang, M., and Huganir, R.L. (2015). Rapid dispersion of SynGAP from synaptic spines triggers AMPA receptor insertion and spine enlargement during LTP. Neuron *85*, 173-189.

Arrazola, M.S., Varela-Nallar, L., Colombres, M., Toledo, E.M., Cruzat, F., Pavez, L., Assar, R., Aravena, A., Gonzalez, M., Montecino, M.*, et al.* (2009). Calcium/calmodulin-dependent protein kinase type IV is a target gene of the Wnt/beta-catenin signaling pathway. Journal of cellular physiology *221*, 658-667.

Ataman, B., Ashley, J., Gorczyca, M., Ramachandran, P., Fouquet, W., Sigrist, S.J., and Budnik, V. (2008). Rapid activity-dependent modifications in synaptic structure and function require bidirectional Wnt signaling. Neuron *57*, 705-718.

Barria, A., Muller, D., Derkach, V., Griffith, L.C., and Soderling, T.R. (1997). Regulatory phosphorylation of AMPA-type glutamate receptors by CaM-KII during long-term potentiation. Science *276*, 2042-2045.

Bassani, S., Folci, A., Zapata, J., and Passafaro, M. (2013). AMPAR trafficking in synapse maturation and plasticity. Cellular and molecular life sciences : CMLS *70*, 4411-4430.

Beaumont, V., Thompson, S.A., Choudhry, F., Nuthall, H., Glantschnig, H., Lipfert, L., David, G.R., Swain, C.J., McAllister, G., and Munoz-Sanjuan, I. (2007). Evidence for an enhancement of excitatory transmission in adult CNS by Wnt signaling pathway modulation. Molecular and cellular neurosciences *35*, 513-524.

Bevins, R.A., and Besheer, J. (2006). Object recognition in rats and mice: a one-trial non-matching-to-sample learning task to study 'recognition memory'. Nature protocols *1*, 1306-1311.

Bian, W.J., Miao, W.Y., He, S.J., Wan, Z.F., Luo, Z.G., and Yu, X. (2015). A novel Wnt5a-Frizzled4 signaling pathway mediates activity-independent dendrite morphogenesis via the distal PDZ motif of Frizzled 4. Developmental neurobiology *75*, 805-822.

Bilic, J., Huang, Y.L., Davidson, G., Zimmermann, T., Cruciat, C.M., Bienz, M., and Niehrs, C. (2007). Wnt induces LRP6 signalosomes and promotes dishevelled-dependent LRP6 phosphorylation. Science *316*, 1619-1622.

Blakely, B.D., Bye, C.R., Fernando, C.V., Horne, M.K., Macheda, M.L., Stacker, S.A., Arenas, E., and Parish, C.L. (2011). Wnt5a regulates midbrain dopaminergic axon growth and guidance. PloS one *6*, e18373.

Bodmer, D., Levine-Wilkinson, S., Richmond, A., Hirsh, S., and Kuruvilla, R. (2009). Wnt5a mediates nerve growth factor-dependent axonal branching and growth in developing sympathetic neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience *29*, 7569-7581.

Boitard, M., Bocchi, R., Egervari, K., Petrenko, V., Viale, B., Gremaud, S., Zgraggen, E., Salmon, P., and Kiss, J.Z. (2015). Wnt signaling regulates multipolar-to-bipolar transition of migrating neurons in the cerebral cortex. Cell reports *10*, 1349-1361.

Bosch, M., and Hayashi, Y. (2012). Structural plasticity of dendritic spines. Current opinion in neurobiology *22*, 383-388.

Broadbelt, K., Byne, W., and Jones, L.B. (2002). Evidence for a decrease in basilar dendrites of pyramidal cells in schizophrenic medial prefrontal cortex. Schizophrenia research *58*, 75-81.

Budnik, V., and Salinas, P.C. (2011). Wnt signaling during synaptic development and plasticity. Current opinion in neurobiology *21*, 151-159.

Castelo-Branco, G., Sousa, K.M., Bryja, V., Pinto, L., Wagner, J., and Arenas, E. (2006). Ventral midbrain glia express region-specific transcription factors and regulate dopaminergic neurogenesis through Wnt-5a secretion. Molecular and cellular neurosciences *31*, 251-262.

Cerpa, W., Gambrill, A., Inestrosa, N.C., and Barria, A. (2011). Regulation of NMDA-receptor synaptic transmission by Wnt signaling. The Journal of neuroscience : the official journal of the Society for Neuroscience *31*, 9466-9471.

Cerpa, W., Godoy, J.A., Alfaro, I., Farias, G.G., Metcalfe, M.J., Fuentealba, R., Bonansco, C., and Inestrosa, N.C. (2008). Wnt-7a modulates the synaptic vesicle cycle and synaptic transmission in hippocampal neurons. The Journal of biological chemistry *283*, 5918-5927.

Chen, C., and Tonegawa, S. (1997). Molecular genetic analysis of synaptic plasticity, activity-dependent neural development, learning, and memory in the mammalian brain. Annual review of neuroscience *20*, 157-184.

Chen, J., Park, C.S., and Tang, S.J. (2006). Activity-dependent synaptic Wnt release regulates hippocampal long term potentiation. The Journal of biological chemistry *281*, 11910-11916.

Chow, D.K., Groszer, M., Pribadi, M., Machniki, M., Carmichael, S.T., Liu, X., and Trachtenberg, J.T. (2009). Laminar and compartmental regulation of dendritic growth in mature cortex. Nature neuroscience *12*, 116-118.

Ciani, L., and Salinas, P.C. (2005). WNTs in the vertebrate nervous system: from patterning to neuronal connectivity. Nature reviews Neuroscience *6*, 351-362.

Clarke, G.L., Chen, J., and Nishimune, H. (2012). Presynaptic Active Zone Density during Development and Synaptic Plasticity. Frontiers in molecular neuroscience *5*, 12.

Clevers, H., and Nusse, R. (2012). Wnt/beta-catenin signaling and disease. Cell *149*, 1192-1205.

Cliffe, A., Hamada, F., and Bienz, M. (2003). A role of Dishevelled in relocating Axin to the plasma membrane during wingless signaling. Current biology : CB *13*, 960-966.

Cline, H., and Haas, K. (2008). The regulation of dendritic arbor development and plasticity by glutamatergic synaptic input: a review of the synaptotrophic hypothesis. The Journal of physiology *586*, 1509-1517.

Cole, A.J., Abu-Shakra, S., Saffen, D.W., Baraban, J.M., and Worley, P.F. (1990). Rapid rise in transcription factor mRNAs in rat brain after electroshock-induced seizures. Journal of neurochemistry *55*, 1920-1927.

Cuitino, L., Godoy, J.A., Farias, G.G., Couve, A., Bonansco, C., Fuenzalida, M., and Inestrosa, N.C. (2010). Wnt-5a modulates recycling of functional GABAA receptors on hippocampal neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience *30*, 8411-8420.

DasGupta, R., and Fuchs, E. (1999). Multiple roles for activated LEF/TCF transcription complexes during hair follicle development and differentiation. Development *126*, 4557-4568.

Davidson, G., and Niehrs, C. (2010). Emerging links between CDK cell cycle regulators and Wnt signaling. Trends in cell biology *20*, 453-460.

Davis, E.K., Zou, Y., and Ghosh, A. (2008). Wnts acting through canonical and noncanonical signaling pathways exert opposite effects on hippocampal synapse formation. Neural development *3*, 32.

De Ferrari, G.V., and Inestrosa, N.C. (2000). Wnt signaling function in Alzheimer's disease. Brain research Brain research reviews *33*, 1-12.

De Ferrari, G.V., Papassotiropoulos, A., Biechele, T., Wavrant De-Vrieze, F., Avila, M.E., Major, M.B., Myers, A., Saez, K., Henriquez, J.P., Zhao, A., *et al.* (2007). Common genetic variation within the low-density lipoprotein receptor-related protein 6 and late-onset Alzheimer's disease. Proceedings of the National Academy of Sciences of the United States of America *104*, 9434-9439.

Derkach, V., Barria, A., and Soderling, T.R. (1999). Ca2+/calmodulin-kinase II enhances channel conductance of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate type glutamate receptors. Proceedings of the National Academy of Sciences of the United States of America *96*, 3269-3274.

Dosemeci, A., and Jaffe, H. (2010). Regulation of phosphorylation at the postsynaptic density during different activity states of Ca2+/calmodulin-dependent protein kinase II. Biochemical and biophysical research communications *391*, 78-84.

Dudek, S.M., and Bear, M.F. (1992). Homosynaptic long-term depression in area CA1 of hippocampus and effects of N-methyl-D-aspartate receptor blockade. Proceedings of the National Academy of Sciences of the United States of America *89*, 4363-4367.

Emoto, K. (2012). Signaling mechanisms that coordinate the development and maintenance of dendritic fields. Current opinion in neurobiology *22*, 805-811.

Farias, G.G., Alfaro, I.E., Cerpa, W., Grabowski, C.P., Godoy, J.A., Bonansco, C., and Inestrosa, N.C. (2009). Wnt-5a/JNK signaling promotes the clustering of PSD-95 in hippocampal neurons. The Journal of biological chemistry *284*, 15857-15866.

Feldmeyer, D., and Radnikow, G. (2009). Developmental alterations in the functional properties of excitatory neocortical synapses. The Journal of physiology *587*, 1889-1896.

Flood, D.G. (1991). Region-specific stability of dendritic extent in normal human aging and regression in Alzheimer's disease. II. Subiculum. Brain research *540*, 83-95.

Fortress, A.M., Schram, S.L., Tuscher, J.J., and Frick, K.M. (2013). Canonical Wnt signaling is necessary for object recognition memory consolidation. The Journal of neuroscience : the official journal of the Society for Neuroscience *33*, 12619-12626.

Fradkin, L.G., Dura, J.M., and Noordermeer, J.N. (2010). Ryks: new partners for Wnts in the developing and regenerating nervous system. Trends in neurosciences *33*, 84-92.

Fukaya, M., Kato, A., Lovett, C., Tonegawa, S., and Watanabe, M. (2003). Retention of NMDA receptor NR2 subunits in the lumen of endoplasmic reticulum in targeted NR1 knockout mice. Proceedings of the National Academy of Sciences of the United States of America *100*, 4855-4860.

Gao, C., and Chen, Y.G. (2010). Dishevelled: The hub of Wnt signaling. Cellular signalling *22*, 717-727.

Gogolla, N., Galimberti, I., Deguchi, Y., and Caroni, P. (2009). Wnt signaling mediates experience-related regulation of synapse numbers and mossy fiber connectivities in the adult hippocampus. Neuron *62*, 510-525.

Gordon, M.D., and Nusse, R. (2006). Wnt signaling: multiple pathways, multiple receptors, and multiple transcription factors. The Journal of biological chemistry *281*, 22429-22433.

Gorski, J.A., Zeiler, S.R., Tamowski, S., and Jones, K.R. (2003). Brain-derived neurotrophic factor is required for the maintenance of cortical dendrites. The Journal of neuroscience : the official journal of the Society for Neuroscience *23*, 6856-6865.

Grumolato, L., Liu, G., Mong, P., Mudbhary, R., Biswas, R., Arroyave, R., Vijayakumar, S., Economides, A.N., and Aaronson, S.A. (2010). Canonical and noncanonical Wnts use a common mechanism to activate completely unrelated coreceptors. Genes & development *24*, 2517-2530.

Guler, A.D., Ecker, J.L., Lall, G.S., Haq, S., Altimus, C.M., Liao, H.W., Barnard, A.R., Cahill, H., Badea, T.C., Zhao, H*., et al.* (2008). Melanopsin cells are the principal conduits for rod-cone input to non-image-forming vision. Nature *453*, 102-105.

Hall, A.C., Lucas, F.R., and Salinas, P.C. (2000). Axonal remodeling and synaptic differentiation in the cerebellum is regulated by WNT-7a signaling. Cell *100*, 525-535.

Heiman, M.G., and Shaham, S. (2010). Twigs into branches: how a filopodium becomes a dendrite. Current opinion in neurobiology *20*, 86-91.

Hodar, C., Assar, R., Colombres, M., Aravena, A., Pavez, L., Gonzalez, M., Martinez, S., Inestrosa, N.C., and Maass, A. (2010). Genome-wide identification of new Wnt/beta-catenin target genes in the human genome using CART method. BMC genomics *11*, 348.

Hua, J.Y., and Smith, S.J. (2004). Neural activity and the dynamics of central nervous system development. Nature neuroscience *7*, 327-332.

Hur, E.M., and Zhou, F.Q. (2010). GSK3 signalling in neural development. Nature reviews Neuroscience *11*, 539-551.

Inestrosa, N.C., and Arenas, E. (2010). Emerging roles of Wnts in the adult nervous system. Nature reviews Neuroscience *11*, 77-86.

Jensen, M., Hoerndli, F.J., Brockie, P.J., Wang, R., Johnson, E., Maxfield, D., Francis, M.M., Madsen, D.M., and Maricq, A.V. (2012). Wnt signaling regulates acetylcholine

receptor translocation and synaptic plasticity in the adult nervous system. Cell *149*, 173-187.

Kaufmann, W.E., and Moser, H.W. (2000). Dendritic anomalies in disorders associated with mental retardation. Cerebral cortex *10*, 981-991.

Keeble, T.R., Halford, M.M., Seaman, C., Kee, N., Macheda, M., Anderson, R.B., Stacker, S.A., and Cooper, H.M. (2006). The Wnt receptor Ryk is required for Wnt5a-mediated axon guidance on the contralateral side of the corpus callosum. The Journal of neuroscience : the official journal of the Society for Neuroscience *26*, 5840-5848.

Kerchner, G.A., and Nicoll, R.A. (2008). Silent synapses and the emergence of a postsynaptic mechanism for LTP. Nature reviews Neuroscience *9*, 813-825.

Kessels, H.W., and Malinow, R. (2009). Synaptic AMPA receptor plasticity and behavior. Neuron *61*, 340-350.

Kikuchi, A., Yamamoto, H., Sato, A., and Matsumoto, S. (2011). New insights into the mechanism of Wnt signaling pathway activation. International review of cell and molecular biology *291*, 21-71.

Kim, M.S., Akhtar, M.W., Adachi, M., Mahgoub, M., Bassel-Duby, R., Kavalali, E.T., Olson, E.N., and Monteggia, L.M. (2012). An essential role for histone deacetylase 4 in synaptic plasticity and memory formation. The Journal of neuroscience : the official journal of the Society for Neuroscience *32*, 10879-10886.

Kohn, A.D., and Moon, R.T. (2005). Wnt and calcium signaling: beta-catenin-independent pathways. Cell calcium *38*, 439-446.

Koleske, A.J. (2013). Molecular mechanisms of dendrite stability. Nature reviews Neuroscience *14*, 536-550.

Korkut, C., and Budnik, V. (2009). WNTs tune up the neuromuscular junction. Nature reviews Neuroscience *10*, 627-634.

Kuhl, M., Sheldahl, L.C., Malbon, C.C., and Moon, R.T. (2000a). Ca(2+)/calmodulin-dependent protein kinase II is stimulated by Wnt and Frizzled homologs and promotes ventral cell fates in Xenopus. The Journal of biological chemistry *275*, 12701-12711.

Kuhl, M., Sheldahl, L.C., Park, M., Miller, J.R., and Moon, R.T. (2000b). The Wnt/Ca2+ pathway: a new vertebrate Wnt signaling pathway takes shape. Trends in genetics : TIG *16*, 279-283.

Kulkarni, V.A., and Firestein, B.L. (2012). The dendritic tree and brain disorders. Molecular and cellular neurosciences *50*, 10-20.

La Porta, C.A., and Comolli, R. (1998). PKC-dependent modulation of IkB alpha-NFkB pathway in low metastatic B16F1 murine melanoma cells and in highly metastatic BL6 cells. Anticancer research *18*, 2591-2597.

Lee, H.K. (2006). AMPA Receptor Phosphorylation in Synaptic Plasticity: Insights from Knockin Mice. In The Dynamic Synapse: Molecular Methods in Ionotropic Receptor Biology, J.T. Kittler, and S.J. Moss, eds. (Boca Raton (FL)).

Lee, H.K., Takamiya, K., Han, J.S., Man, H., Kim, C.H., Rumbaugh, G., Yu, S., Ding, L., He, C., Petralia, R.S.*, et al.* (2003). Phosphorylation of the AMPA receptor GluR1 subunit is required for synaptic plasticity and retention of spatial memory. Cell *112*, 631-643.

Lee, S.J., Escobedo-Lozoya, Y., Szatmari, E.M., and Yasuda, R. (2009). Activation of CaMKII in single dendritic spines during long-term potentiation. Nature *458*, 299-304.

LeGates, T.A., Altimus, C.M., Wang, H., Lee, H.K., Yang, S., Zhao, H., Kirkwood, A., Weber, E.T., and Hattar, S. (2012). Aberrant light directly impairs mood and learning through melanopsin-expressing neurons. Nature *491*, 594-598.

Li, L., Hutchins, B.I., and Kalil, K. (2009). Wnt5a induces simultaneous cortical axon outgrowth and repulsive axon guidance through distinct signaling mechanisms. The Journal of neuroscience : the official journal of the Society for Neuroscience *29*, 5873-5883.

Lie, D.C., Colamarino, S.A., Song, H.J., Desire, L., Mira, H., Consiglio, A., Lein, E.S., Jessberger, S., Lansford, H., Dearie, A.R.*, et al.* (2005). Wnt signalling regulates adult hippocampal neurogenesis. Nature *437*, 1370-1375.

Linden, D.J. (2012). A late phase of LTD in cultured cerebellar Purkinje cells requires persistent dynamin-mediated endocytosis. Journal of neurophysiology *107*, 448-454.

Lisman, J., Schulman, H., and Cline, H. (2002). The molecular basis of CaMKII function in synaptic and behavioural memory. Nature reviews Neuroscience *3*, 175-190.

Lisman, J., Yasuda, R., and Raghavachari, S. (2012). Mechanisms of CaMKII action in long-term potentiation. Nature reviews Neuroscience *13*, 169-182.

Liu, C.C., Tsai, C.W., Deak, F., Rogers, J., Penuliar, M., Sung, Y.M., Maher, J.N., Fu, Y., Li, X., Xu, H.*, et al.* (2014). Deficiency in LRP6-mediated Wnt signaling contributes to synaptic abnormalities and amyloid pathology in Alzheimer's disease. Neuron *84*, 63-77.

Logan, C.Y., and Nusse, R. (2004). The Wnt signaling pathway in development and disease. Annual review of cell and developmental biology *20*, 781-810.

Lu, W., and Roche, K.W. (2012). Posttranslational regulation of AMPA receptor trafficking and function. Current opinion in neurobiology *22*, 470-479.

Lu, W., Shi, Y., Jackson, A.C., Bjorgan, K., During, M.J., Sprengel, R., Seeburg, P.H., and Nicoll, R.A. (2009). Subunit composition of synaptic AMPA receptors revealed by a single-cell genetic approach. Neuron *62*, 254-268.

Lucas, F.R., and Salinas, P.C. (1997). WNT-7a induces axonal remodeling and increases synapsin I levels in cerebellar neurons. Developmental biology *192*, 31-44.

Luo, L. (2002). Actin cytoskeleton regulation in neuronal morphogenesis and structural plasticity. Annual review of cell and developmental biology *18*, 601-635.

Lyford, G.L., Yamagata, K., Kaufmann, W.E., Barnes, C.A., Sanders, L.K., Copeland, N.G., Gilbert, D.J., Jenkins, N.A., Lanahan, A.A., and Worley, P.F. (1995). Arc, a growth factor and activity-regulated gene, encodes a novel cytoskeleton-associated protein that is enriched in neuronal dendrites. Neuron *14*, 433-445.

Ma, H., Groth, R.D., Cohen, S.M., Emery, J.F., Li, B., Hoedt, E., Zhang, G., Neubert, T.A., and Tsien, R.W. (2014). gammaCaMKII shuttles Ca(2)(+)/CaM to the nucleus to trigger CREB phosphorylation and gene expression. Cell *159*, 281-294.

Ma, H., Groth, R.D., Wheeler, D.G., Barrett, C.F., and Tsien, R.W. (2011). Excitation-transcription coupling in sympathetic neurons and the molecular mechanism of its initiation. Neuroscience research *70*, 2-8.

MacDonald, B.T., Tamai, K., and He, X. (2009). Wnt/beta-catenin signaling: components, mechanisms, and diseases. Developmental cell *17*, 9-26.

Maguschak, K.A., and Ressler, K.J. (2011). Wnt signaling in amygdala-dependent learning and memory. The Journal of neuroscience : the official journal of the Society for Neuroscience *31*, 13057-13067.

Malenka, R.C., Kauer, J.A., Perkel, D.J., Mauk, M.D., Kelly, P.T., Nicoll, R.A., and Waxham, M.N. (1989). An essential role for postsynaptic calmodulin and protein kinase activity in long-term potentiation. Nature *340*, 554-557.

Malinow, R., and Malenka, R.C. (2002). AMPA receptor trafficking and synaptic plasticity. Annual review of neuroscience *25*, 103-126.

Malinow, R., Schulman, H., and Tsien, R.W. (1989). Inhibition of postsynaptic PKC or CaMKII blocks induction but not expression of LTP. Science *245*, 862-866.

Mammen, A.L., Kameyama, K., Roche, K.W., and Huganir, R.L. (1997). Phosphorylation of the alpha-amino-3-hydroxy-5-methylisoxazole4-propionic acid receptor GluR1 subunit by calcium/calmodulin-dependent kinase II. The Journal of biological chemistry *272*, 32528-32533.

Matter, C., Pribadi, M., Liu, X., and Trachtenberg, J.T. (2009). Delta-catenin is required for the maintenance of neural structure and function in mature cortex in vivo. Neuron *64*, 320-327.

McAllister, A.K. (2007). Dynamic aspects of CNS synapse formation. Annual review of neuroscience *30*, 425-450.

Milat, F., and Ng, K.W. (2009). Is Wnt signalling the final common pathway leading to bone formation? Molecular and cellular endocrinology *310*, 52-62.

Montcouquiol, M., Crenshaw, E.B., 3rd, and Kelley, M.W. (2006). Noncanonical Wnt signaling and neural polarity. Annual review of neuroscience *29*, 363-386.

Mulkey, R.M., and Malenka, R.C. (1992). Mechanisms underlying induction of homosynaptic long-term depression in area CA1 of the hippocampus. Neuron *9*, 967-975.

Murakoshi, H., Wang, H., and Yasuda, R. (2011). Local, persistent activation of Rho GTPases during plasticity of single dendritic spines. Nature *472*, 100-104.

Nakayama, A.Y., Harms, M.B., and Luo, L. (2000). Small GTPases Rac and Rho in the maintenance of dendritic spines and branches in hippocampal pyramidal neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience *20*, 5329-5338.

Neves, G., Cooke, S.F., and Bliss, T.V. (2008). Synaptic plasticity, memory and the hippocampus: a neural network approach to causality. Nature reviews Neuroscience *9*, 65-75.

Newpher, T.M., and Ehlers, M.D. (2008). Glutamate receptor dynamics in dendritic microdomains. Neuron *58*, 472-497.

Nicoll, R.A., and Roche, K.W. (2013). Long-term potentiation: peeling the onion. Neuropharmacology *74*, 18-22.

Nikopoulos, K., Venselaar, H., Collin, R.W., Riveiro-Alvarez, R., Boonstra, F.N., Hooymans, J.M., Mukhopadhyay, A., Shears, D., van Bers, M., de Wijs, I.J.*, et al.* (2010). Overview of the mutation spectrum in familial exudative vitreoretinopathy and Norrie disease with identification of 21 novel variants in FZD4, LRP5, and NDP. Human mutation *31*, 656-666.

Nusse, R., and Varmus, H. (2012). Three decades of Wnts: a personal perspective on how a scientific field developed. The EMBO journal *31*, 2670-2684.

Oliva, C.A., Vargas, J.Y., and Inestrosa, N.C. (2013). Wnt signaling: role in LTP, neural networks and memory. Ageing research reviews *12*, 786-800.

Onishi, K., Hollis, E., and Zou, Y. (2014). Axon guidance and injury-lessons from Wnts and Wnt signaling. Current opinion in neurobiology *27*, 232-240.

Packard, M., Koo, E.S., Gorczyca, M., Sharpe, J., Cumberledge, S., and Budnik, V. (2002). The Drosophila Wnt, wingless, provides an essential signal for pre- and postsynaptic differentiation. Cell *111*, 319-330.

Petersen, C.P., and Reddien, P.W. (2009). Wnt signaling and the polarity of the primary body axis. Cell *139*, 1056-1068.

Pokorny, J., and Yamamoto, T. (1981a). Postnatal ontogenesis of hippocampal CA1 area in rats. I. Development of dendritic arborisation in pyramidal neurons. Brain research bulletin *7*, 113-120.

Pokorny, J., and Yamamoto, T. (1981b). Postnatal ontogenesis of hippocampal CA1 area in rats. II. Development of ultrastructure in stratum lacunosum and moleculare. Brain research bulletin *7*, 121-130.

Polakis, P. (2012). Wnt signaling in cancer. Cold Spring Harbor perspectives in biology *4*.

Ramanan, N., Shen, Y., Sarsfield, S., Lemberger, T., Schutz, G., Linden, D.J., and Ginty, D.D. (2005). SRF mediates activity-induced gene expression and synaptic plasticity but not neuronal viability. Nature neuroscience *8*, 759-767.

Roche, K.W., O'Brien, R.J., Mammen, A.L., Bernhardt, J., and Huganir, R.L. (1996). Characterization of multiple phosphorylation sites on the AMPA receptor GluR1 subunit. Neuron *16*, 1179-1188.

Rodriguez-Gil, D.J., and Greer, C.A. (2008). Wnt/Frizzled family members mediate olfactory sensory neuron axon extension. The Journal of comparative neurology *511*, 301-317.

Rosso, S.B., Sussman, D., Wynshaw-Boris, A., and Salinas, P.C. (2005). Wnt signaling through Dishevelled, Rac and JNK regulates dendritic development. Nature neuroscience *8*, 34-42.

Ryu, Y.K., Collins, S.E., Ho, H.Y., Zhao, H., and Kuruvilla, R. (2013). An autocrine Wnt5a-Ror signaling loop mediates sympathetic target innervation. Developmental biology *377*, 79-89.

Safholm, A., Tuomela, J., Rosenkvist, J., Dejmek, J., Harkonen, P., and Andersson, T. (2008). The Wnt-5a-derived hexapeptide Foxy-5 inhibits breast cancer metastasis in vivo by targeting cell motility. Clinical cancer research : an official journal of the American Association for Cancer Research *14*, 6556-6563.

Sahores, M., Gibb, A., and Salinas, P.C. (2010). Frizzled-5, a receptor for the synaptic organizer Wnt7a, regulates activity-mediated synaptogenesis. Development *137*, 2215-2225.

Salinas, P.C., and Zou, Y. (2008). Wnt signaling in neural circuit assembly. Annual review of neuroscience *31*, 339-358.

Sanes, D.H., and Kotak, V.C. (2011). Developmental plasticity of auditory cortical inhibitory synapses. Hearing research *279*, 140-148.

Saneyoshi, T., Wayman, G., Fortin, D., Davare, M., Hoshi, N., Nozaki, N., Natsume, T., and Soderling, T.R. (2008). Activity-dependent synaptogenesis: regulation by a CaM-kinase kinase/CaM-kinase I/betaPIX signaling complex. Neuron *57*, 94-107.

Schwarz-Romond, T., Metcalfe, C., and Bienz, M. (2007). Dynamic recruitment of axin by Dishevelled protein assemblies. Journal of cell science *120*, 2402-2412.

Seeburg, P.H. (1993). The TINS/TiPS Lecture. The molecular biology of mammalian glutamate receptor channels. Trends in neurosciences *16*, 359-365.

Sfakianos, M.K., Eisman, A., Gourley, S.L., Bradley, W.D., Scheetz, A.J., Settleman, J., Taylor, J.R., Greer, C.A., Williamson, A., and Koleske, A.J. (2007). Inhibition of Rho via Arg and p190RhoGAP in the postnatal mouse hippocampus regulates dendritic spine maturation, synapse and dendrite stability, and behavior. The Journal of neuroscience : the official journal of the Society for Neuroscience *27*, 10982-10992.

Shafer, B., Onishi, K., Lo, C., Colakoglu, G., and Zou, Y. (2011). Vangl2 promotes Wnt/planar cell polarity-like signaling by antagonizing Dvl1-mediated feedback inhibition in growth cone guidance. Developmental cell *20*, 177-191.

Sharma, K., Choi, S.Y., Zhang, Y., Nieland, T.J., Long, S., Li, M., and Huganir, R.L. (2013). High-throughput genetic screen for synaptogenic factors: identification of LRP6 as critical for excitatory synapse development. Cell reports *5*, 1330-1341.

Sheldahl, L.C., Park, M., Malbon, C.C., and Moon, R.T. (1999). Protein kinase C is differentially stimulated by Wnt and Frizzled homologs in a G-protein-dependent manner. Current biology : CB *9*, 695-698.

Shepherd, J.D., and Huganir, R.L. (2007). The cell biology of synaptic plasticity: AMPA receptor trafficking. Annual review of cell and developmental biology *23*, 613-643.

Shimogori, T., VanSant, J., Paik, E., and Grove, E.A. (2004). Members of the Wnt, Fz, and Frp gene families expressed in postnatal mouse cerebral cortex. The Journal of comparative neurology *473*, 496-510.

Slusarski, D.C., Corces, V.G., and Moon, R.T. (1997a). Interaction of Wnt and a Frizzled homologue triggers G-protein-linked phosphatidylinositol signalling. Nature *390*, 410-413.

Slusarski, D.C., Yang-Snyder, J., Busa, W.B., and Moon, R.T. (1997b). Modulation of embryonic intracellular Ca2+ signaling by Wnt-5A. Developmental biology *182*, 114-120.

Spinsanti, P., De Vita, T., Caruso, A., Melchiorri, D., Misasi, R., Caricasole, A., and Nicoletti, F. (2008). Differential activation of the calcium/protein kinase C and the canonical beta-catenin pathway by Wnt1 and Wnt7a produces opposite effects on cell proliferation in PC12 cells. Journal of neurochemistry *104*, 1588-1598.

Stanfield, B.B., and Cowan, W.M. (1979). The development of the hippocampus and dentate gyrus in normal and reeler mice. The Journal of comparative neurology *185*, 423-459.

Sweatt, J.D. (1999). Toward a molecular explanation for long-term potentiation. Learning & memory *6*, 399-416.

Tabatadze, N., Tomas, C., McGonigal, R., Lin, B., Schook, A., and Routtenberg, A. (2012). Wnt transmembrane signaling and long-term spatial memory. Hippocampus *22*, 1228-1241.

Tang, S.J. (2014). Synaptic activity-regulated Wnt signaling in synaptic plasticity, glial function and chronic pain. CNS & neurological disorders drug targets *13*, 737-744.

Tolias, K.F., Bikoff, J.B., Burette, A., Paradis, S., Harrar, D., Tavazoie, S., Weinberg, R.J., and Greenberg, M.E. (2005). The Rac1-GEF Tiam1 couples the NMDA receptor to the activity-dependent development of dendritic arbors and spines. Neuron *45*, 525-538.

Traynelis, S.F., Wollmuth, L.P., McBain, C.J., Menniti, F.S., Vance, K.M., Ogden, K.K., Hansen, K.B., Yuan, H., Myers, S.J., and Dingledine, R. (2010). Glutamate receptor ion channels: structure, regulation, and function. Pharmacological reviews *62*, 405-496.

Tronche, F., Kellendonk, C., Kretz, O., Gass, P., Anlag, K., Orban, P.C., Bock, R., Klein, R., and Schutz, G. (1999). Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety. Nature genetics *23*, 99-103.

Tsien, J.Z., Chen, D.F., Gerber, D., Tom, C., Mercer, E.H., Anderson, D.J., Mayford, M., Kandel, E.R., and Tonegawa, S. (1996a). Subregion- and cell type-restricted gene knockout in mouse brain. Cell *87*, 1317-1326.

Tsien, J.Z., Huerta, P.T., and Tonegawa, S. (1996b). The essential role of hippocampal CA1 NMDA receptor-dependent synaptic plasticity in spatial memory. Cell *87*, 1327-1338.

Ultanir, S.K., Kim, J.E., Hall, B.J., Deerinck, T., Ellisman, M., and Ghosh, A. (2007). Regulation of spine morphology and spine density by NMDA receptor signaling in vivo. Proceedings of the National Academy of Sciences of the United States of America *104*, 19553-19558.

Valnegri, P., Puram, S.V., and Bonni, A. (2015). Regulation of dendrite morphogenesis by extrinsic cues. Trends in neurosciences *38*, 439-447.

Van Aelst, L., and Cline, H.T. (2004). Rho GTPases and activity-dependent dendrite development. Current opinion in neurobiology *14*, 297-304.

van Amerongen, R., and Nusse, R. (2009). Towards an integrated view of Wnt signaling in development. Development *136*, 3205-3214.

Varela-Nallar, L., Alfaro, I.E., Serrano, F.G., Parodi, J., and Inestrosa, N.C. (2010a). Wingless-type family member 5A (Wnt-5a) stimulates synaptic differentiation and function of glutamatergic synapses. Proceedings of the National Academy of Sciences of the United States of America *107*, 21164-21169.

Varela-Nallar, L., Aranguiz, F.C., Abbott, A.C., Slater, P.G., and Inestrosa, N.C. (2010b). Adult hippocampal neurogenesis in aging and Alzheimer's disease. Birth defects research Part C, Embryo today : reviews *90*, 284-296.

Vargas, J.Y., Fuenzalida, M., and Inestrosa, N.C. (2014). In vivo activation of Wnt signaling pathway enhances cognitive function of adult mice and reverses cognitive deficits in an Alzheimer's disease model. The Journal of neuroscience : the official journal of the Society for Neuroscience *34*, 2191-2202.

Veeman, M.T., Axelrod, J.D., and Moon, R.T. (2003). A second canon. Functions and mechanisms of beta-catenin-independent Wnt signaling. Developmental cell *5*, 367-377.

Vorhees, C.V., and Williams, M.T. (2006). Morris water maze: procedures for assessing spatial and related forms of learning and memory. Nature protocols *1*, 848-858.

Walsh, D.M., and Selkoe, D.J. (2004). Deciphering the molecular basis of memory failure in Alzheimer's disease. Neuron *44*, 181-193.

Wayman, G.A., Impey, S., Marks, D., Saneyoshi, T., Grant, W.F., Derkach, V., and Soderling, T.R. (2006). Activity-dependent dendritic arborization mediated by CaM-kinase I activation and enhanced CREB-dependent transcription of Wnt-2. Neuron *50*, 897-909.

Wayman, G.A., Lee, Y.S., Tokumitsu, H., Silva, A.J., and Soderling, T.R. (2008). Calmodulin-kinases: modulators of neuronal development and plasticity. Neuron *59*, 914-931.

Welters, H.J., and Kulkarni, R.N. (2008). Wnt signaling: relevance to beta-cell biology and diabetes. Trends in endocrinology and metabolism: TEM *19*, 349-355.

Wenthold, R.J., Petralia, R.S., Blahos, J., II, and Niedzielski, A.S. (1996). Evidence for multiple AMPA receptor complexes in hippocampal CA1/CA2 neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience *16*, 1982-1989.

West, A.E., and Greenberg, M.E. (2011). Neuronal activity-regulated gene transcription in synapse development and cognitive function. Cold Spring Harbor perspectives in biology *3*.

Whitlock, J.R., Heynen, A.J., Shuler, M.G., and Bear, M.F. (2006). Learning induces long-term potentiation in the hippocampus. Science *313*, 1093-1097.

Wong, R.O., and Ghosh, A. (2002). Activity-dependent regulation of dendritic growth and patterning. Nature reviews Neuroscience *3*, 803-812.

Wyllie, D.J., and Nicoll, R.A. (1994). A role for protein kinases and phosphatases in the Ca(2+)-induced enhancement of hippocampal AMPA receptor-mediated synaptic responses. Neuron *13*, 635-643.

Xie, Y., Hayden, M.R., and Xu, B. (2010). BDNF overexpression in the forebrain rescues Huntington's disease phenotypes in YAC128 mice. The Journal of neuroscience : the official journal of the Society for Neuroscience *30*, 14708-14718.

Xie, Z., Srivastava, D.P., Photowala, H., Kai, L., Cahill, M.E., Woolfrey, K.M., Shum, C.Y., Surmeier, D.J., and Penzes, P. (2007). Kalirin-7 controls activity-dependent structural and functional plasticity of dendritic spines. Neuron *56*, 640-656.

Xu, B., Zang, K., Ruff, N.L., Zhang, Y.A., McConnell, S.K., Stryker, M.P., and Reichardt, L.F. (2000). Cortical degeneration in the absence of neurotrophin signaling: dendritic retraction and neuronal loss after removal of the receptor TrkB. Neuron *26*, 233-245.

Yamaguchi, T.P., Bradley, A., McMahon, A.P., and Jones, S. (1999). A Wnt5a pathway underlies outgrowth of multiple structures in the vertebrate embryo. Development *126*, 1211-1223.

Yan, D., Wiesmann, M., Rohan, M., Chan, V., Jefferson, A.B., Guo, L., Sakamoto, D., Caothien, R.H., Fuller, J.H., Reinhard, C.*, et al.* (2001). Elevated expression of axin2 and hnkd mRNA provides evidence that Wnt/beta -catenin signaling is activated in human colon tumors. Proceedings of the National Academy of Sciences of the United States of America *98*, 14973-14978.

Yang, G., Pan, F., and Gan, W.B. (2009). Stably maintained dendritic spines are associated with lifelong memories. Nature *462*, 920-924.

Yoshimura, Y., Shinkawa, T., Taoka, M., Kobayashi, K., Isobe, T., and Yamauchi, T. (2002). Identification of protein substrates of Ca(2+)/calmodulin-dependent protein kinase II in the postsynaptic density by protein sequencing and mass spectrometry. Biochemical and biophysical research communications *290*, 948-954.

Yu, X., and Malenka, R.C. (2003). Beta-catenin is critical for dendritic morphogenesis. Nature neuroscience *6*, 1169-1177.

Zhang, X., Zhu, J., Yang, G.Y., Wang, Q.J., Qian, L., Chen, Y.M., Chen, F., Tao, Y., Hu, H.S., Wang, T.*, et al.* (2007). Dishevelled promotes axon differentiation by regulating atypical protein kinase C. Nature cell biology *9*, 743-754.

# Curriculum Vitae

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## Education

**Johns Hopkins University** **Baltimore, MD**
Ph.D. Department of Biology 09/2009~12/2016
Thesis: Wnt5a is essential for dendrite maintenance and cognitive functions in the adult brain

**National Chiao Tung University** **Hsinchu, Taiwan**
M.S. Institute of Biological Science and Technology 09/1998~06//2000
Honor: Ministry of Education Scholarship for Graduate Students
Thesis: Expression and Purification of the Recombinant Porcine Lactoferrin.

**National Taiwan Ocean University** **Keelung, Taiwan**
B.S. Department of Food Science 09/1994~06/1998

## Publication

Chiu SL, **Chen CM**, Cline HT. (2008) Insulin receptor signaling regulates synapse number, dendritic plasticity and circuit function *in vivo*. *Neuron* 58, 708-719.

**Chen CM***, Chiu SL*, Cline HT. (2009) Co-expression of Argonaute2 enhances short hairpin RNA-induced RNA interference in *Xenopus* CNS neurons *in vivo*. *Frontiers in Neural Circuits*. 3, 1-13. * Co-first authors.

Thomas GM, Hayashi T, Chiu SL, **Chen CM**, Huganir RL. (2012) Palmitoylation by DHHC5/8 targets GRIP1 to dendritic endosomes to regulate AMPA-R trafficking. *Neuron* 73(3):482-96

Anggono V, Koç-Schmitz Y, Widagdo J, Kormann J, Quan A, **Chen CM**, Robinson PJ, Choi SY, Linden DJ, Plomann M, Huganir RL. (2013) PICK1 interacts with PACSIN to regulate AMPA receptor internalization and cerebellar long-term dedpression. *Proc Natl Acad Sci* 110(34):13976-81.

## Research and Experience

**Graduate Student in Prof. Rejji Kuruvilla lab** 09/2009~12/2016
Discover roles of Wnt5a signaling in synaptic plasticity and functions in the hippocampal dependent learning and memory.

Identify essential role of Wnt5a for neuronal morphologies in adult hippocampus

Uncover Wnt signaling pathways important for synaptic plasticity and dendritic maintenance

**Lab technician volunteer in Prof. Richard Huganir lab** 11/2008~08/2009
Assistant with structure and function analysis in PICK1 and PACSIN interaction for AMPA receptor internalization

Assistant with generating Palmitoylation deficient DHHC5/8 lenti virus for AMPA receptor trafficking

**Lab technician in Prof. Hollis Cline lab** 09/2004~09/2008
Build up vector based RNAi system in *Xenopus laevis* neurons

Identify insulin receptors signaling in *Xenopus* brain

**Lab technician in Dr. Senthil K. Muthuswamy lab** 09/2003~09/2004
To build up hetero-dimerization systems for EGF receptors family

To generate mammary stable cell lines for studying roles of EGF receptor dimers in breast cancer